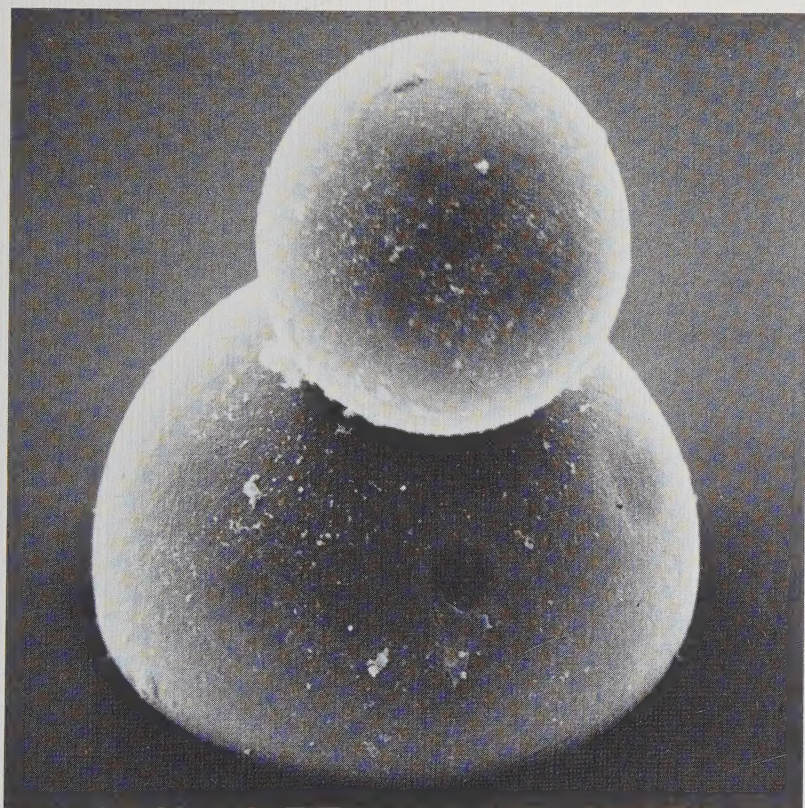
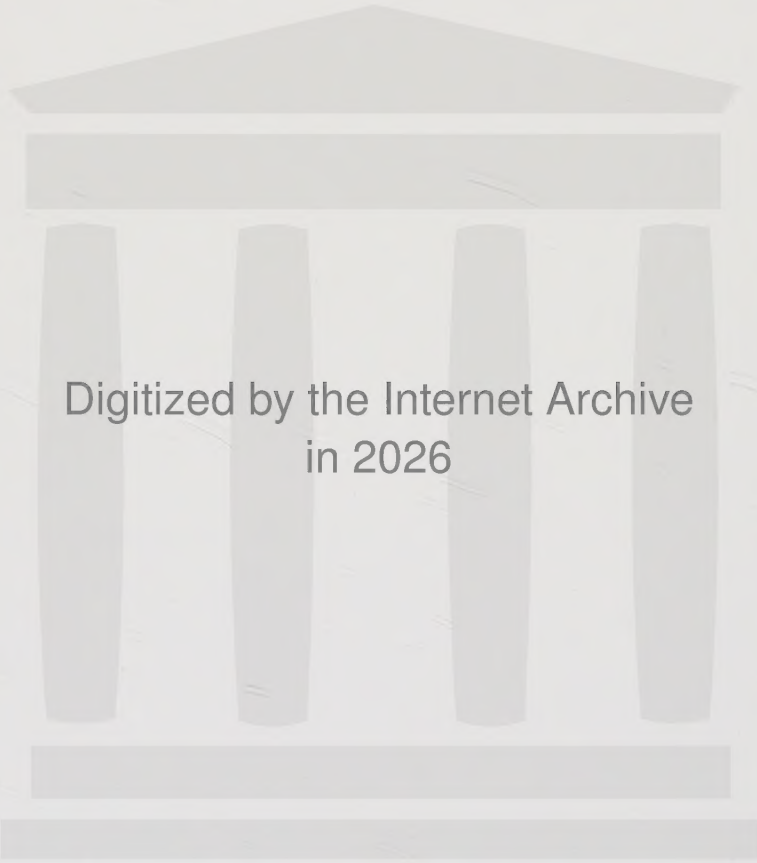


# Studies on the Short-term Regulation of Lipolysis in Rat Adipocytes with Special Regard to the Anti-lipolytic Effect of Insulin



Nils Östen Nilsson

Lund 1981



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STUDIES ON THE SHORT-TERM REGULATION OF  
LIPOLYSIS IN RAT ADIPOCYTES WITH  
SPECIAL REGARD TO THE ANTI-LIPOLYTIC EFFECT OF INSULIN

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Nils Östen Nilsson

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Lund 1981



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| <b>Title and subtitle</b><br>Studies on the short-term regulation of lipolysis in rat adipocytes with special regard to the anti-lipolytic effect of insulin.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |                                               |                              |
| <b>Abstract</b><br>Phosphorylation of hormone-sensitive lipase (HSL), the rate-controlling enzyme in adipose tissue lipolysis, has been demonstrated in intact rat adipocytes. Methods have been developed to determine the state of HSL phosphorylation in parallel with its activity in isolated fat cells. HSL activity was continuously followed by pH-stat titration of released fatty acids. Noradrenaline stimulated lipolysis through increased HSL phosphorylation, as verified by the close temporal and quantitative relationship between these two parameters. Incubations with dibutyryl cyclic AMP, determination of intracellular cyclic AMP and data obtained by others on the phosphorylation of isolated HSL provided strong evidence that the noradrenaline effect was mediated through activation of cyclic AMP-dependent protein kinase, in accordance with the cyclic AMP-second messenger hypothesis. There was no indication of an additional cyclic AMP-independent 'substrate activation' mechanism involved in the noradrenaline activation of the lipolysis. Insulin inhibited lipolysis by preventing and/or decreasing the noradrenaline-stimulated increase of the HSL phosphorylation state, as demonstrated by the close temporal and quantitative relationship between these parameters. These effects were facilitated by adenosine, endogenously produced from the adipocytes during the incubations. Insulin had no effect on maximal dibutyryl cyclic AMP stimulation of HSL phosphorylation and HSL activity but prevented most of the noradrenaline-induced cyclic AMP elevation. These effects, in comparison with those of agents known to inhibit cyclic AMP production, indicated that insulin exerted most of its anti-lipolytic action through reduction of cyclic AMP, while some of its effect may be due to cyclic AMP-independent mechanisms. A hypothetical model for the mode of insulin action on lipolysis is presented. Acetate inhibited lipolysis after submaximal noradrenaline stimulation, most likely through a mechanism which did not involve a change of the HSL phosphorylation state. |                                               |                              |
| <b>Key words</b><br>Isolated rat adipocytes, hormone-sensitive lipase, phosphorylation state, FFA release, pH-stat titration, noradrenaline, insulin, cyclic AMP, dibutyryl cyclic AMP, adenosine, acetate, mechanism of action.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |                                               |                              |
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Nils Östen Nilsson

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1981-05-13

From the Department of Physiological Chemistry  
University of Lund, Sweden

STUDIES ON THE SHORT-TERM REGULATION OF  
LIPOLYSIS IN RAT ADIPOCYTES WITH  
SPECIAL REGARD TO THE ANTI-LIPOLYTIC EFFECT OF INSULIN

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The cover illustration is a scanning electron micrograph (1200 x magnification) of isolated rat adipocytes used in the study. For preparation details see the legend to Fig. 1.

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To

Eli, Andreas and Henrik





The thesis is based on the following papers, which will be referred to by their Roman numerals:

- I. Nilsson, N.Ö. and Belfrage, P. 1978  
Effects of acetate, acetaldehyde, and ethanol on lipolysis in isolated rat adipocytes.  
*J. Lipid Res.* 19:737-741
- II. Nilsson, N.Ö. and Belfrage P. 1979  
Continuous monitoring of free fatty acid release from adipocytes by pH-stat titration.  
*J. Lipid Res.* 20:557-560
- III. Nilsson, N.Ö. and Belfrage, P. 1981  
Continuous measurement of free fatty acid release from intact adipocytes by pH-stat titration.  
In *Methods in Enzymology* (Lowenstein, J.M. ed.) Vol. 72 [20]:319-325. Academic Press, New York.
- IV. Belfrage, P., Fredrikson, G., Nilsson, N.Ö. and Strålfors, P. 1980  
Regulation of adipose tissue lipolysis: Phosphorylation of hormone-sensitive lipase in intact rat adipocytes.  
*FEBS Lett.* 111:120-124
- V. Nilsson, N.Ö., Strålfors, P., Fredrikson, G. and Belfrage, P. 1980  
Regulation of adipose tissue lipolysis: Effects of nor-adrenaline and insulin on phosphorylation of hormone-sensitive lipase and on lipolysis in intact rat adipocytes.  
*FEBS Lett.* 111:125-130
- VI. Nilsson, N.Ö. and Belfrage, P. 1981  
The anti-lipolytic effect of insulin: Inhibition of lipolysis in rat adipocytes by decreased extent of phosphorylation of hormone-sensitive lipase.  
*Manuscript.*

VII. Nilsson, N.Ö. and Belfrage, P. 1981

The anti-lipolytic effect of insulin: Time-course relationships between cyclic adenosine 3':5'-monophosphate levels, phosphorylation state of hormone-sensitive lipase and lipolysis in rat adipocytes.

*Manuscript.*

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## ABBREVIATIONS

Ado, adenosine; ATP, adenosine 5'-triphosphate; BSA, bovine serum albumin; cyclic AMP, cyclic adenosine 3':5'-monophosphate; FFA, free (non-esterified) fatty acids; HSL, hormone-sensitive lipase; INS, insulin; NA, noradrenaline; PCV, packed cell volume; SDS-PAGE, polyacrylamide gel electrophoresis in presence of sodium dodecyl sulphate; TG, triacylglycerols.



## GENERAL INTRODUCTION

Lipid molecules are energy-rich and their hydrophobic character allow them to be densely packed in the absence of water. Fat cells, adipocytes, are specialized for lipid and thus energy storage, so that the triacylglycerols packed in a large central lipid droplet constitute 70-80% of the wet weight of the cells. Since adipose tissue is mainly composed of adipocytes and humans normally have 10-20 kg of the tissue, the energy depot is large. Fig. 1 demonstrates the dense packing of adipocytes in rat adipose tissue and the cover illustration isolated fat cells from rat adipose tissue used in this study.

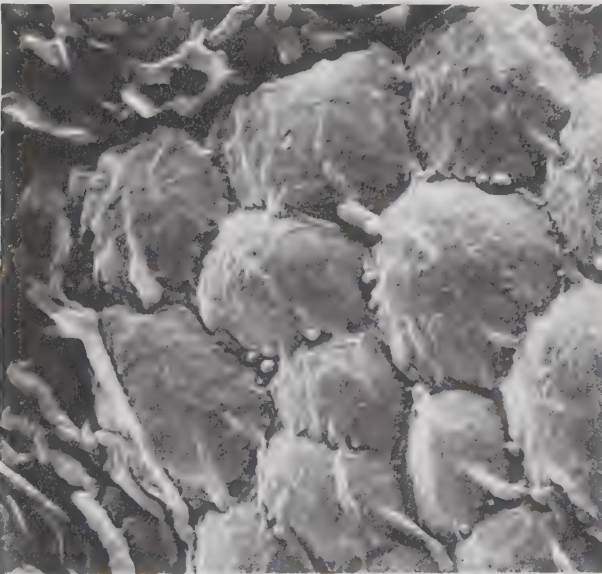


Fig. 1. Scanning electron micrograph (450 x magnification) of rat epididymal adipose tissue with several densely packed adipocytes protruding from the mesenterical surface of the specimen.

The specimen in this Fig. and that of the cover were fixed in osmium tetroxide and glutaraldehyde, dehydrated with ethanol and critical-point dried. The isolated cells were applied to wax and both specimens covered by gold. The micrographs were taken with a Stereoscan scanning electron microscope.

Adipose tissue rapidly assimilates fatty acids provided by the lipoprotein lipase catalyzed hydrolysis of lipoprotein triacylglycerols at the capillary endothelium. The lipoproteins derive from the lipid absorption in the gastrointestinal tract

(chylomicrons) and from the liver (very low density lipoproteins, VLDL). Plasma insulin levels rise after a meal and stimulate lipogenesis by activating the lipoprotein lipase and the uptake of glucose necessary for esterification of the fatty acids.

Adipose tissue lipolysis, in the following referred to as lipolysis, is the process by which the adipocyte triacylglycerols are enzymatically degraded to glycerol and free fatty acids (FFA). The short-term regulation of this process is the main subject for this thesis. The FFA are insoluble in the blood plasma but become bound to albumin and transported to muscle, liver and other organs. The flow of FFA from the adipose tissue and VLDL from the liver provides a mechanism for energy transport to different organs. During 'fight and flight' reactions catecholamine release in seconds or minutes causes activation of lipolysis thus providing energy mainly intended for muscle work. The increased insulin level during and after a meal inhibits lipolysis and facilitates storage. During fasting the decreasing insulin level successively relieves the inhibition so that the stored energy can be utilized.

## BACKGROUND

### Free fatty acids (FFA)

Laurell (1956) first suggested that fat might be transported from the fat depots in the plasma as albumin-bound FFA and described the increased plasma FFA concentration during prolonged starvation and diabetic ketoacidosis and the decrease caused by insulin administration. Studies by several workers (Dole, 1956; Gordon and Cherkes, 1956; Laurell, 1957) then established the fundamental importance of this lipid transport form.

The quantity of energy supplied to the organism by FFA is high because of the high fractional disappearance rate, approxima-

tely 0.25, although the plasma concentration is low (0.1-0.8 mM) (Scow and Chernick, 1970).

The ability of albumin to bind FFA decreases exponentially as the FFA to albumin molar ratio (normally around 1 to 2), increases. This probably facilitates the accessibility at the various organs (Scow and Chernick, 1970). The FFA uptake by the tissues is directly related to its plasma concentration and the blood flow to a particular organ. The rate of FFA mobilization in adipose tissue controls the plasma FFA concentration and thus largely determines the tissue FFA uptake. The FFA mobilization from adipose tissue is regulated by the nutritional state and by hormonal and neural mechanisms.

The FFA release rate is the net result of the lipolysis and the reesterification of part of the FFA to adipocyte triacylglycerol. The latter process may be significant under certain conditions, especially at high glucose and insulin concentrations, but the rate of FFA mobilization generally reflects the rate of lipolysis. Thus, the regulation of this process is likely to normally determine the FFA mobilization rate.

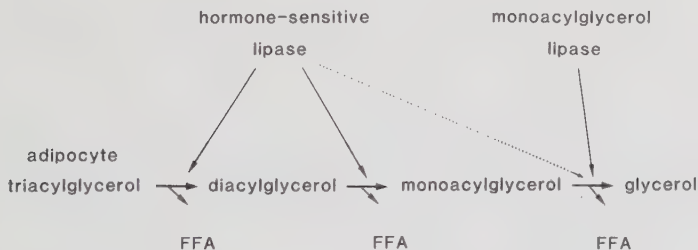


Fig. 2. Postulated roles of enzymes catalyzing adipose tissue lipolysis. Hydrolysis of the first ester-bond catalyzed by the hormone-sensitive lipase is rate-limiting.

### Hormone-sensitive lipase

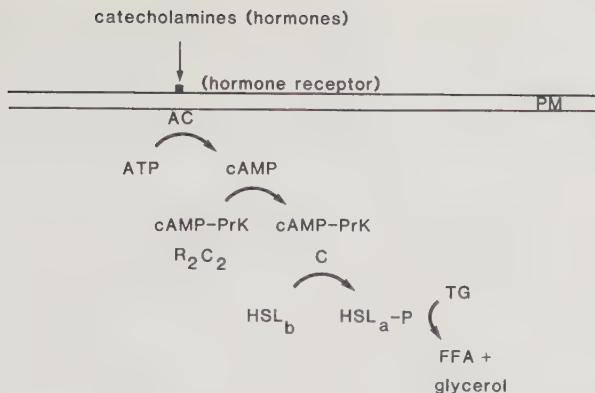
The sequential degradation of triacylglycerol to FFA and glycerol during adipose tissue lipolysis is illustrated in Fig. 2. The rate-controlling enzyme, the hormone-sensitive lipase (HSL) was first characterized by Vaughan *et al.* (1964), but

the existence of a hormonally controlled lipolytic activity had been indicated previously (Hollenberg *et al.*, 1961; Rizack, 1961; Björntorp and Furman, 1962; Vaughan and Steinberg, 1963). The HSL catalyzes the hydrolysis of the first (rate-limiting) and the second ester bond, but the monoacylglycerols (Tornqvist *et al.*, 1978) may be hydrolyzed mainly by a separate monoacylglycerol lipase (Tornqvist and Belfrage, 1976). This enzyme is not regulated by hormones or nutritional state.

The hormonal regulation of HSL was early proposed to be mediated by cyclic AMP-dependent phosphorylation, in analogy with the hormonal regulation of glycogen degradation (Rizack, 1964; Vaughan *et al.*, 1964). A mechanism for hormonal activation of HSL (see below, Fig. 3) was subsequently postulated, mainly based on the work by Steinberg and collaborators (Steinberg *et al.*, 1975). However, since HSL was very difficult to purify, direct experimental evidence for the proposed regulation mechanism could not be obtained, until the enzyme had been successfully solubilized and identified with a particular polypeptide on SDS-PAGE (Belfrage *et al.*, 1977). It was subsequently purified extensively and partially characterized (Fredrikson *et al.*, 1981). The purified HSL had an apparent minimum  $M_r=84,000$ , catalyzed hydrolysis of emulsified tri-, di- and monooleoylglycerol and cholesterol oleate at the relative rates of 1:10:4:1.5 with high molar specific activity, and was phosphorylated and thereby activated by the catalytic subunit of cyclic AMP-dependent protein kinase and ATP-Mg (Fredrikson *et al.*, 1981).

### Short-term regulation of lipolysis

A large number of reports have been published regarding the regulation of lipolysis in adipose tissue and isolated adipocytes; for details see recent reviews (Severson, 1979; Fain, 1980). In the following the presentation will be restricted to short-term regulation. Studies during the last 15 years have resulted in the postulated mechanism illustrated in Fig. 3 for the activation of lipolysis by a number of lipolytic



**Fig. 3.** Simplified model of the hormonal activation cascade for HSL. The interaction between the hormone and its receptor results in activation of adenylate cyclase in the plasma membrane. Adenylate cyclase catalyzes the formation of cyclic AMP, which functions as a second messenger, transferring the message from the hormone (first messenger) at the cell surface, via an activation of a protein kinase to the HSL, the target enzyme. HSL becomes phosphorylated and thereby activated, increasing the rate of lipolysis. The reactions constitute an amplification system since the hormones are only present at pico- or nanomolar concentrations, while cyclic AMP and protein kinase are in the micromolar range. PM, plasma membrane; AC,adenylate-cyclase; cAMP-PrK, cyclic AMP-dependent protein kinase ( $R_2C_2$ =inactive holoenzyme with two regulatory and two catalytic subunits; C=active catalytic subunit). Cyclic AMP binding to R results in the dissociation of the holoenzyme);  $HSL_b$ , nonphosphorylated, subactive HSL;  $HSL_a-P$ , phosphorylated, active HSL; TG, triacylglycerol.

hormones - the 'lipolytic activation cascade' (Steinberg *et al.*, 1975). The evidence for this postulated mechanism was entirely based on enzyme activity data, until recently when the purified HSL could be shown to become phosphorylated and activated by cyclic AMP-dependent protein kinase (see above). However, HSL phosphorylation and activation in the intact fat cell had not been demonstrated when the present investigation was performed.

While there has thus been consensus on a common, postulated mechanism for lipolysis activation, the anti-lipolytic action of insulin, one of the most important effects of this hormone, has been the subject of considerable controversy. Jungas and Ball (1963) were the first to demonstrate inhibition of lipolysis by insulin in intact adipose tissue, as did Fain *et al.* (1966) in isolated rat adipocytes. *In vivo* evidence for the



anti-lipolytic effect of insulin are provided from diabetic ketoacidosis where the elevated FFA concentration immediately starts to decrease upon infusion of the hormone (Scow and Chernick, 1970). The same effect was noted already in the previously cited work by Laurell (1956).

Possible modes of action for the anti-lipolytic effect of insulin have been discussed in a number of reviews (Fain, 1974; Jungas, 1975; Steinberg *et al.*, 1975; Czech, 1977; Severson, 1979; Fain, 1980) and only a brief summary will be made in this connection. The inhibition of lipolysis has been shown to involve decreased HSL activity suggested to be due to a block in the conversion of HSL to HSL<sub>a</sub>-P (Fig. 3) or, possibly, by an increased HSL deactivation through increased lipase phosphatase activity (Steinberg *et al.*, 1975). Evidence for protein phosphatase-catalyzed deactivation of HSL in cell-free preparations were obtained in several studies by Steinberg and collaborators (Khoo *et al.*, 1976; Severson *et al.*, 1977).

In early studies the mechanism for the insulin effect was believed to be a reduction of intracellular cyclic AMP (Butcher *et al.*, 1966; Butcher *et al.*, 1968; Desai *et al.*, 1973; Kono and Barham, 1973; Siddle and Hales, 1974) mediated through stimulation of low  $K_m$  phosphodiesterase activity (Loten and Sneyd, 1970; Manganiello and Vaughan, 1973) or possibly adenylylate cyclase inhibition (Hepp, 1972). However, several subsequent studies indicated that the insulin inhibition could not be accounted for by the reduction observed in total cyclic AMP (Fain and Rosenberg, 1972; Khoo *et al.*, 1973; Knight and Iliffe, 1973; Steinberg *et al.*, 1975). Recent studies (Steinberg *et al.*, 1975; Wieser and Fain, 1975) and reviews (Czech, 1977; Fain, 1980) have tended to emphasize cyclic AMP-independent mechanisms for the anti-lipolytic action of insulin, although the possibility that cellular cyclic AMP is compartmentalized (Jarett *et al.*, 1972; Corbin *et al.*, 1977; Hayes *et al.*, 1979; Hayes *et al.*, 1980) has left the whole question unanswered at the time of the present investigation.

The hormonal regulation of lipolysis can be modulated in different ways. Adenosine is released both *in vivo* (Fredholm and Sollevi, 1981) and from isolated adipocytes (Schwabe *et al.*, 1973), and is a potent inhibitor of adenylate cyclase (for review see Fain and Malbon, 1979). Adenosine has been suggested to facilitate insulin action on several processes in adipocytes, including cyclic AMP accumulation and lipolysis (Schwabe *et al.*, 1974). Adenosine deaminase converts adenosine to inosine, and thereby inactivates it (Fain and Malbon, 1979).

Methylxanthines, *e.g.* theophylline, stimulate lipolysis via increased cyclic AMP probably through competition with adenosine and, at high levels, by inhibition of phosphodiesterase (*cf.* Hjemsdahl and Sollevi, 1978).

Free fatty acids are effective feed-back inhibitors of lipolysis when the FFA to albumin molar ratio exceeds 2-3. The effect is probably mediated both through inhibition of adenylate cyclase and through a direct effect on HSL (Fain and Shepherd, 1976).

#### PURPOSE AND DESIGN OF THE INVESTIGATION

Mechanisms for hormonal short-term regulation of lipolysis in intact fat cells were investigated in this thesis. Answers to the following main questions were sought:

1. Do lipolytic hormones activate lipolysis through an increase in the phosphorylation state of HSL?
2. If so, through which mechanism?
3. Does insulin exert its anti-lipolytic action through a decrease in the phosphorylation state of HSL?
4. If so, through which mechanism?

The basic methodological approach was incubations in a model system of isolated rat adipocytes. Methods were developed for continuous determination of lipolysis, directly reflecting the biological activity of HSL, and parallel analysis of the state of phosphorylation of HSL in the adipocytes.

## RESULTS

Preparation of and incubation with intact rat adipocytes  
(I, II, III, V, VI)

The use of intact adipocytes, prepared by collagenase digestion as originally described by Rodbell (1964), has several advantages over work with intact adipose tissue. A homogeneous cell preparation is obtained, diffusion problems are avoided and a large number of experiments can be performed under identical conditions with cells from the same batch. Conditions will be different from those existing in intact tissue, which will certainly influence at least quantitative responses to hormones and other agents. However, basal mechanisms for hormonal action like those studied in the present investigation, are likely to be the same. An additional factor which must be taken into consideration is the mode of incubation used. In this study adipocytes have been incubated in an accumulating system; thus, changing concentrations of *e.g.* FFA and adenosine, must be taken into account when interpreting the results.

Rat fat cells are easy to prepare by collagenase digestion, but a number of methodological pitfalls exist which can lead to highly variable hormone sensitivity, as illustrated below. Detailed procedures for the preparation of the fat cells used in this study are given in papers I-III. Several factors are critical for retaining cell intactness: collagenase and albumin batches, collagenase concentration, incubation time and type, intensity of stirring during collagenase digestion, handling of cells during the isolation procedure and use of plastic or siliconized glass vials.

Fat from rats weighing 120-150 g were chosen because their adipocytes were smaller and therefore more resistant to mechanical damage. The hormonal sensitivity and maximal response changes with rat age or cell size. Therefore, the rat weight was kept within strict limits. Measures were also taken to assure that all rats were in the same nutritional state when sacrificed for cell preparation. Since thus the cell size was fairly constant from batch to batch (range of diameters 47-53

$\mu\text{m}$ ) results could be related to the packed cell volume (PCV), which was simply and reliably determined in microhematocrite centrifuge tubes in each preparation (*cf.* III). If medium trapped between the cells (about 1%) was neglected, one  $\mu\text{l}$  of PCV corresponded to  $1.58 \cdot 10^4 \pm 0.23 \cdot 10^4$  cells from rats of weight  $137 \pm 12.6$  g ; mean  $\pm$  SD,  $n=10$ ). One ml PCV of fat cells corresponded to approx. 0.7 g lipids, and 4 mg of protein (determined according to Lowry *et al.* (1951)). The intactness of cells was evaluated by the microscopic appearance, the amount of broken cells as indicated by a free fat layer in the microhematocrite centrifugation tube, and by the ability of insulin to stimulate their glucose utilization as described in paper III.

Incubation conditions have been described in detail in the various papers.

Hormone sensitivity of adipocytes used in other studies have varied considerably. For comparison some examples will be given and related to rat weight, cell concentration and albumin concentration, since these factors can influence lipolysis.

Hormonal sensitivity of fat cells used is illustrated in Fig. 4, with insulin inhibition of maximally noradrenaline-stimulated fat cells (half-maximal inhibition at 25 pM), insulin stimulation of glucose utilization under identical conditions (half-maximal at 90 pM) and noradrenaline stimulation under somewhat different conditions (half-maximal at 25 nM). In all three cases hormonal sensitivity is high, in approximately the same concentration range as expected to be found for serum insulin and noradrenaline under physiological conditions. The lipolytic response to noradrenaline in a work by Fain and Shepherd (1976), (120-160 g rats, 27 mg cells/ml, 30 mg/ml BSA) had an  $\text{ED}_{50}$  value (effective dose that gives 50% of the maximal value obtainable) of 0.55  $\mu\text{M}$  and a maximal lipolysis corresponding to about 1.6  $\mu\text{mol}$  FFA/ml PCV/min, (*cf.* the values in this work:  $\text{ED}_{50} = 0.03$   $\mu\text{M}$  and max lipolysis 1.5-2.2  $\mu\text{mol}$  FFA/ml PCV/min). From the data of Thomas *et al.*

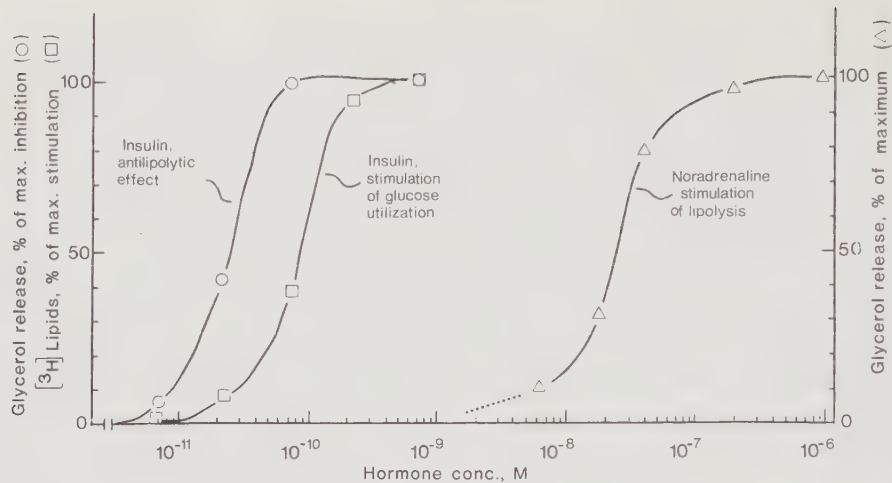


Fig. 4. Hormone sensitivity of isolated rat adipocytes used in the study, illustrated by representative dose response curves for

- the antilipolytic effect of insulin measured as inhibition of glycerol release
- the insulin stimulation of glucose uptake and conversion to cellular lipids
- the lipolytic effect of noradrenaline measured as glycerol release.

Incubation details can be found in papers I and VI. Normal levels in human serum are 30-1000 pM insulin and less than 10 nM catecholamines.

(1979) (100-200 g rats,  $1 \cdot 10^5 - 2 \cdot 10^5$  cells/2 ml, 30 mg/ml BSA) it could be calculated that the adrenaline  $ED_{50}$  was 2  $\mu$ M and max. lipolysis corresponded to about 0.82  $\mu$ mol/ml PCV/min calculated from the highest concentration used (50  $\mu$ M). The sensitivity of the anti-lipolytic effect of insulin in the same work was, however, high with  $ED_{50}$  values of 20-30 pM insulin (*cf.* Fig. 4, 25 pM). The effect of insulin on glucose utilization which had  $ED_{50}$  values of 50 pM ( $1.6 \cdot 10^5$  cells/ml) (II,III) to 90 pM ( $8 \cdot 10^5$  cells/ml) (VI), in early works by Rodbell (1964) (30-40  $\mu$ mol cell triacylglycerol/ml) was about 90 pM. The same parameter was 55-90 pM in a study with cells of different sizes (Foley *et al.*, 1980) with the lower value for small cells. Schwabe *et al.* (1974), (140-160 g rats, 1-2  $\mu$ l PCV/ml, 20 mg/ml BSA) got  $ED_{50} = 0.5 \mu$ M for noradrenaline stimulation. In the same work the insulin  $ED_{50}$  was 350 pM at 0.3  $\mu$ l PCV/ml and 140 pM at 6  $\mu$ l PCV/ml; the differences indicate adenosine effects that will be discussed. Green and News-holme (1979) (100-140 g rats, 32  $\mu$ l PCV/ml, 4% BSA) obtained



equal insulin  $ED_{50}$  values, about 35 pM, for both stimulation of glucose utilization and lipolysis inhibition. Adenosine was shown to markedly shift the dose-response curve for glucose utilization to the left. Kono and Barham (1973) presented data where the insulin  $ED_{50}$  value for inhibition of noradrenaline-stimulated (100  $\mu$ M) lipolysis was approximately 500 pM.

Anti-lipolytic activity of adenosine also differed in various studies. Under the conditions used in paper VI a 50% reduction (at submaximal noradrenaline stimulation) was achieved with 10-50 nM adenosine. In adipocytes perfusion systems where endogenous adenosine is constantly removed, Turpin *et al.* (1977) found a 50% reduction in adrenaline-stimulated lipolysis by 10 nM adenosine while Hjemdahl and Sollevi (1978) reduced noradrenaline-stimulated lipolysis 25% by 1  $\mu$ M adenosine.

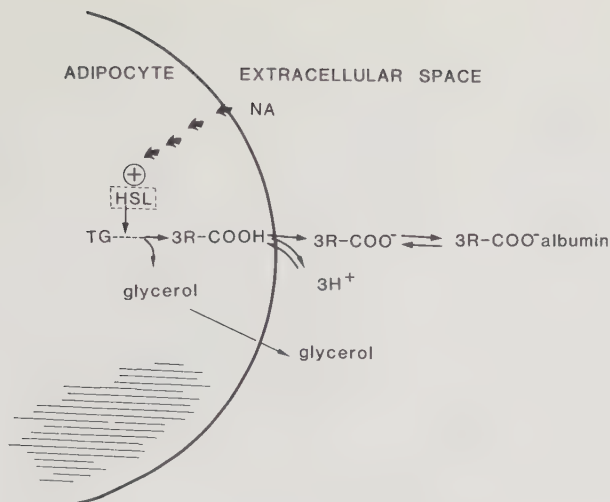
From the comparison above it is obvious that the hormonal sensitivity of the fat cells used in this study was equal or higher than that obtained in several recent works with isolated rat adipocytes. The lower sensitivity has in several cases forced other workers to stimulate the cells with much higher, hormone concentrations. The reason for the lower hormone sensitivity is probably insufficiently controlled preparation technique, but also incubation conditions; notably cell concentration and accumulation of adenosine plays an important role (*cf.* Schwabe *et al.*, 1974).

#### Determination of the rate of lipolysis (I,II,III)

Lipolysis rate was measured either as glycerol or FFA release. Glycerol release directly reflects the rate of lipolysis, since little glycerol re-utilization occurs in adipocytes (Rodbell, 1965). FFA release, on the other hand, reflects the net result of lipolysis and re-esterification, as discussed above, and therefore could not unconditionally be used as a measure of lipolysis. However, in isolated rat adipocytes little re-esterification takes place, if the FFA/albumin ratio is kept low (Rodbell, 1965). In glucose-free incubation medium FFA and glycerol were released at a molar three-to-one ratio

(II) indicating insignificant re-esterification. Even in the presence of 5 mM glucose, a high insulin concentration and after a long incubation glycerol and FFA data obtained indicated that less than 15% of the FFA could have been re-esterified (unpublished observation). Therefore FFA or glycerol release have been used alternatively throughout this study as a measure of the lipolysis rate. Since HSL is the enzyme controlling the rate of lipolysis (see above) the lipolysis rate directly reflects the HSL activity in the intact fat cells.

In paper I an enzymatic fluorometric method for glycerol analysis developed by others (Laurell and Tibbling, 1965) was modified to obtain higher sensitivity, which was important in that study with submaximal noradrenaline stimulation. In short, dilution of samples were minimized by changing the amount of  $\text{ZnSO}_4$  and  $\text{Ba(OH)}_2$ , used for protein precipitation, and background fluorescence was minimized by modification of the concentration of different reagents. Nevertheless, short-time kinetic studies were difficult to perform with this assay. Therefore a new technique was developed based on the fact that FFA release from fat cells is accompanied by an equivalent proton release (Rudman and Shank, 1966) (Fig. 5). By the use of an automatic pH-stat titrator, it was possible to continuously follow the lipolysis rate as the proton release. Since the pH-stat itself provided the buffering function, buffers were not necessary. Details on the method are given in paper II and III. One could argue that the method is not specific for FFA but would also measure lactate, which is produced by adipocytes (Flatt and Ball, 1964). However, in the absence of glucose and with adequate oxygen supply to the incubations, lactate production was less than 3% of the basal FFA release. In the presence of 5 mM glucose lactate production was 10-15% of the basal FFA release and about 2% of maximal noradrenaline stimulated lipolysis (unpublished observations) indicating that lactate production was insignificant under the conditions of the present study.



**Fig. 5.** The principle for pH-stat titration of FFA release. The FFA release is accompanied by a stoichiometric amount of protons. This is registered by a pH electrode coupled to a pH-stat apparatus which titrates the protons released continuously and stoichiometrically. The titration rate and thus the FFA release rate is monitored on a recorder. The sensitivity of the method is increased by low buffering capacity of the medium, the pH-stat serving as the buffer. With two identical set-ups of apparatus controls and hormone effects can be studied in parallel under identical conditions. R = hydrocarbon chain of fatty acids.

The great advantage of the pH-stat titration technique for determination of the lipolysis rate is the ease of its operation and the possibility to follow the detailed time-course of hormone effects, even within seconds of hormone addition. Since the time-course of the parameters studied in this work (cyclic AMP, HSL phosphorylation state, lipolysis rate) vary rapidly and differently to hormonal perturbation, this possibility is a prerequisite for meaningful studies of their interrelation. A considerable part of the varying relations between cyclic AMP and lipolysis reported in the literature may possibly be accounted for by not taking this into consideration.

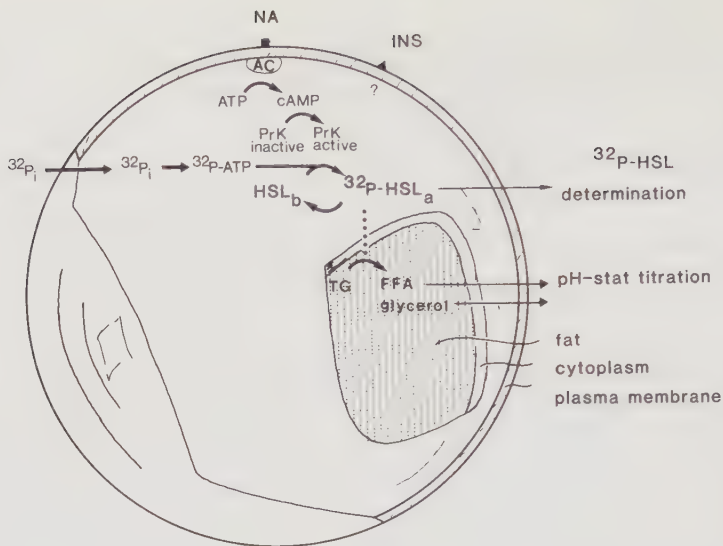


Fig. 6. Experimental approach for the study of HSL phosphorylation in intact fat cells. Adipocytes are incubated in a medium with [ $^{32}\text{P}$ ]orthophosphate ( $^{32}\text{P}_i$ ) which is taken up by the cells and labelling the ATP pool to a constant specific activity. Then, hormones are added, lipolysis is monitored by pH-stat titration and, from the same incubation, samples are taken for analysis of the extent of  $^{32}\text{P}$ -phosphorylation of HSL or other parameters. Thus parallel determinations of the HSL phosphorylation state and of the biological activity of the enzyme can be made. Abbreviations and symbols as in Fig. 3.

#### Demonstration of phosphorylation of HSL in intact adipocytes (IV)

In an attempt to demonstrate HSL phosphorylation in intact fat cells isolated adipocytes were incubated with [ $^{32}\text{P}$ ]orthophosphate and stimulated with noradrenaline as described in detail in (IV). If HSL was phosphorylated, transfer of [ $^{32}\text{P}$ ]orthophosphate from the  $\gamma$ -phosphate position of intracellular [ $^{32}\text{P}$ ]ATP to site(s) on the HSL molecule could be expected to take place (Fig. 6). After rapid isolation of the incubated fat cells, the pH 5.2 precipitate fraction, known to contain most of the HSL in adipose tissue, was prepared (Fredrikson *et al.*, 1981). Detergent solubilization and subsequent chromatographies of the pH 5.2 precipitate fraction were then performed by other members of the group essentially according to the preparative purification procedures for HSL (Fredrikson *et al.*, 1981). The iso-

lated HSL-activity correlated well with a  $^{32}\text{P}$ -radioactivity peak in the final chromatography steps. In the finally purified preparation, with HSL at 35% protein purity, conclusive evidence for  $^{32}\text{P}$ -phosphorylation of HSL was obtained, by comparison with pure [ $^{32}\text{P}$ ]HSL references, and with HSL activity data (IV).

#### Determination of the phosphorylation state of HSL in intact adipocytes (V,VI)

The procedure described in the preceding section could be used for demonstration of HSL phosphorylation in intact fat cells, but for obvious reasons was not very useful for routine analysis of the HSL phosphorylation state after hormonal perturbation of the cells. A more direct approach to this problem was offered by the finding that [ $^{32}\text{P}$ ]HSL migrated exactly as a small 84,000 d-[ $^{32}\text{P}$ ]phosphopeptide band on SDS-PAGE slab gels, in an electrophoresis system optimized for best possible resolution in this molecular size region, of defatted adipocyte [ $^{32}\text{P}$ ]phosphopeptides (V). Hormonal alterations of the [ $^{32}\text{P}$ ]phosphate content of this band were apparent. The obviously critical question was whether these alterations reflected changes of the HSL phosphorylation state, or if they were due to some other [ $^{32}\text{P}$ ]phosphopeptide with the same migration rate. Further fractionation of the [ $^{32}\text{P}$ ]phosphopeptides in the 84,000 d band, as SDS-polypeptide complexes after electrophoretic elution from the gel, demonstrated that by far the main part of the [ $^{32}\text{P}$ ]phosphopeptides was due to [ $^{32}\text{P}$ ]HSL and, moreover, that hormonal perturbation almost entirely affected this [ $^{32}\text{P}$ ]phosphopeptide (V). Thus, quantitation of the  $^{32}\text{P}$  in the 84,000 d [ $^{32}\text{P}$ ]phosphopeptide band could be used for analysis of the extent of HSL  $^{32}\text{P}$ -phosphorylation.

If  $^{32}\text{P}$ -phosphorylation of HSL shall represent true phosphate transfer it is required that a constant specific radioactivity of the precursor, the  $\gamma$ - $^{32}\text{P}$ -phosphate of intracellular ATP, has been obtained. It was found that a steady-state level of this parameter was obtained after 30-40 min incubation of the fat cells with [ $^{32}\text{P}$ ]orthophosphate (V), with little change over at least the next 30 min, even after additions of hormones.



Thus, all phosphorylation experiments include a 40 min preincubation period with [ $^{32}\text{P}$ ]orthophosphate before the addition of hormones. After this time point HSL  $^{32}\text{P}$ -phosphorylation changes can be assumed to directly reflect alterations of the HSL phosphorylation state.

At first unexpected, it was found that HSL was  $^{32}\text{P}$ -phosphorylated during this preincubation period, the  $^{32}\text{P}$ -incorporation curve closely following that of [ $^{32}\text{P}$ ]ATP. This HSL  $^{32}\text{P}$ -phosphorylation also reached a steady state level after 30-40 min and was not accompanied by any change of the lipolysis rate (V). Subsequent work (VI) indicated that this 'basal HSL phosphorylation', besides some [ $^{32}\text{P}$ ]phosphate exchange, was probably due to phosphorylation at another site(s) than that (those) involved in the hormonal regulation.

The phosphorylation state of HSL has been expressed in relative terms, either as amount of [ $^{32}\text{P}$ ]HSL in an aliquot of fat cells, or as percentage of the basal HSL phosphorylation state. The minute amounts of HSL protein in the adipocyte samples have so far precluded any direct determination of moles of phosphate per mole of HSL.

#### Effects of noradrenaline and dibutyryl cyclic AMP on the HSL phosphorylation state and on lipolysis rate (V,VI,VII)

Combining the newly developed method for determination of the HSL phosphorylation state and the continuous monitoring of the HSL activity, as the lipolysis rate, the simultaneous effect of noradrenaline on both parameters was studied in intact fat cells.

The basal lipolysis rate in the absence of noradrenaline during the 40 min preincubation period was low ( $< 0.03 \mu\text{mol FFA released/min/ml PCV}$ ). Addition of noradrenaline rapidly increased the lipolysis rate several-fold to a maximal value of approx.  $2 \mu\text{mol/min/ml PCV}$  (IV). The high sensitivity of the fat cells to noradrenaline (half-maximal effect at 30 nM) has been discussed above (Fig. 4).

An important new result of the investigation was the finding that noradrenaline stimulated lipolysis through an increase of the HSL phosphorylation state (V-VII). The causal relation between the two parameters was demonstrated by their close temporal (V-VII) as well as quantitative relationship (V,VII). This relationship has been an invariable finding in a large number of experiments over several years. Blocking of the noradrenaline activation of the  $\beta$ -adrenoceptors with the  $\beta$ -blocking agent promptly decreased the HSL phosphorylation state and lipolysis rate, indicating the existence of an efficient dephosphorylating system (VII). A protein phosphatase which catalyzes the dephosphorylation and deactivation of isolated HSL has recently been partially purified from rat adipose tissue (H. Olsson, P. Strålfors, and P. Belfrage, unpublished work).

Several observations indicate that noradrenaline increased the HSL phosphorylation state through increased cellular cyclic AMP which activated cyclic AMP-dependent protein kinase, as postulated in the 'lipolytic activation cascade' hypothesis, discussed above. Maximal dibutyryl cyclic AMP stimulation had the same effects on HSL phosphorylation and lipolysis as maximal noradrenaline concentration, but with somewhat different time-course (VI). The most prominent effect of dibutyryl cyclic AMP on isolated fat cells is known to be a marked increase of intracellular cyclic AMP (Jarett and Smith, 1974). Moreover, determinations of cyclic AMP after noradrenaline stimulation directly related this compound to HSL phosphorylation and lipolysis rate in a manner, which was, at least, consistent with a causal relationship (VII). Compartmentalization as an explanation to the finding that the HSL phosphorylation state and the lipolysis rate remained high while cyclic AMP increased transiently and then apparently returned to the basal level has been discussed in (VII).

Isolated HSL has been shown to become phosphorylated and activated by cyclic AMP-dependent protein kinase from rat adipose tissue (Fredrikson *et al.*, 1981). The rate of phosphorylation has recently been demonstrated to be comparable with that found

in the intact cell (P. Strålfors, unpublished work). Nimmo and Cohen (1977) have recently modified the criteria (Krebs, 1973) that should be met before a protein could be said to serve as a physiological substrate for cyclic AMP-dependent protein kinase *in vivo*. These criteria (Nimmo and Cohen, 1977) have now been fulfilled for HSL with the exception that it has not yet been demonstrated, that HSL phosphorylation in intact fat cells occurs at the same site(s) phosphorylated on isolated HSL by cyclic AMP-dependent protein kinase. With this in mind all data so far obtained indicate that noradrenaline stimulates lipolysis through the postulated mechanism (Steinberg *et al.*, 1975), illustrated in Fig. 3.

Based on work by Wise and Jungas (1978) and on the much higher degree of hormonal activation obtained in intact adipocytes than with isolated HSL (Fredrikson *et al.*, 1981) it has been suggested that an additional, cyclic AMP independent 'substrate activation' would operate during noradrenaline stimulation of lipolysis (Fain, 1980). However, although this possibly cannot be definitely ruled out, it does not seem likely for several reasons: The marked hormonal activation of lipolysis in isolated adipocytes is mainly due to the exceptionally low basal lipolysis rate compared to intact tissue (Fredrikson *et al.*, 1981). Dibutyryl cyclic AMP caused the same effects on lipolysis as noradrenaline, through HSL phosphorylations. We have never observed an activation of the lipolysis rate in intact adipocytes that was not correlated to an increase of the HSL phosphorylation state.

Effects of insulin on the HSL phosphorylation state and on the lipolysis rate, and facilitation of these effects by adenosine (II, III, V, VI, VII)

The anti-lipolytic effect of insulin on noradrenaline-stimulated adipocytes could easily be demonstrated in the pH-stat system (II, III, V-VII). As could be expected (Schwabe *et al.*, 1974) with the rather concentrated ( $1.6-8 \cdot 10^5$  cells/ml) cell suspensions that were used, the sensitivity to insulin was high, with half-maximal inhibition in the 10-25 pM range (V,

VI and Fig. 3). Adenosine facilitation of the anti-lipolytic effect by insulin obviously occurred (*cf.* Schwabe *et al.*, 1974) as indicated by the effects of adenosine deaminase and by the shift to the left of the insulin *vs.* anti-lipolysis does-response curve by added adenosine (VI).

The adenosine modification of the effects of insulin can be expected to have varied somewhat between different experiments depending on *e.g.* the cell concentration, albumin batch (since the albumin may contain various amounts of adenosine deaminase (Fain and Malbon, 1979)) and the time of preincubation. This will certainly have influenced the quantitative effects of the hormone additions and can be responsible for part of the variation in hormone sensitivity between different cell batches. Also, the characteristics of the model system used in this investigation, with rather concentrated cell suspensions and thus considerable adenosine accumulation, will be different from an experimental model with dilute cell suspensions or a non-accumulating system. However, as discussed briefly in paper VII, the presently used experimental conditions in several aspects resemble those found in the intact rat adipose tissue. Furthermore, the mechanisms for the hormone effects described in this investigation are likely to be valid also in another experimental model, even if the quantitative effects may differ.

A new result of obvious importance was the finding that insulin exerted its antilipolytic effect by preventing or decreasing the extent of noradrenaline-induced HSL phosphorylation, (V-VII). That the inhibition of lipolysis was indeed caused by the effect on the HSL phosphorylation state was verified by the close temporal and quantitative relationship between the two parameters (V, VI). This insulin effect on HSL phosphorylation state and lipolysis has been an invariable finding in a large number of experiments, under varying conditions. It cannot be explained by changes in specific radioactivity of intracellular [ $^{32}$ P]ATP (V, VI) since this was not significantly changed by insulin addition. Hormones were always added after steady state  $^{32}$ P-phosphorylation of HSL had been obtained, and

insulin addition did not significantly affect this basal HSL phosphorylation state (V,VI). As discussed in a preceding section there is good evidence that the measured  $^{32}\text{P}$ -incorporation into, and  $^{32}\text{P}$ -release from HSL indeed reflects true changes of the HSL phosphorylation state. In addition, the insulin effects on the lipolysis rate have been measured both as glycerol and as FFA release (II,V,VI). As discussed above, under the experimental conditions used, little reesterification occurred and FFA release directly reflected the lipolysis rate. Thus, a number of potential methodological pitfalls have been avoided, substantiating the conclusions drawn from the insulin experiments.

From these experiments no conclusions regarding the underlying mechanism for the insulin effect on the HSL phosphorylation state could be drawn. Thus, it could not be decided if cyclic AMP-dependent or -independent mechanisms were involved or whether the effects resulted from increased dephosphorylation, *i.e.* increased protein phosphatase activity, or decreased phosphorylation, *i.e.* decreased cyclic AMP-dependent protein kinase activity. The results of additional experiments with dibutyryl cyclic AMP (VI) and with determinations of cyclic AMP concentrations (VII) designed to give some further insight into this matter will be discussed in the last section.

#### Inhibition of lipolysis and stimulation of glucose utilization - sensitivity to insulin (VI)

As illustrated above (Fig. 4), and discussed in paper VI the anti-lipolytic effect was almost four-fold more sensitive to insulin than the stimulation of glucose utilization, when measured under identical conditions. The latter parameter was measured as [ $^3\text{H}$ ]glucose conversion to adipocyte lipids and probably mainly reflects the rate of glucose uptake into the cells (Moody *et al.*, 1974). This difference is in accordance with older findings (Fain *et al.*, 1966; Hepp *et al.*, 1967) but more recent studies (Green and Newsholme, 1979; Thomas *et al.*, 1979) seemed to indicate that no significant differences in insulin sensitivity for the two processes existed. However, as



discussed in paper VI, in comparisons of this type it is essential that the experimental conditions really are identical, not just that the same batch of cells was used. At least in the case of the study by Thomas *et al.* (1979) non-identical incubation conditions were used. In the other study (Green and Newsholme, 1979) exact conditions for the experiments are not given and inclusion of adenosine deaminase made the experiment difficult to evaluate.

#### Effects of acetate on HSL phosphorylation state and on lipolysis rate (I)

During the initial part of this study the role of acetate, acetaldehyde and ethanol in the regulation of lipolysis was investigated. The reason for this was the observations, that ethanol intake was accompanied by a decrease in the plasma FFA level (Lieber *et al.*, 1962), and that acetate infusion in man reduced the same parameter (Crouse *et al.*, 1968), suggesting that acetate inhibited lipolysis. Acetate, but not ethanol or acetaldehyde, was found to inhibit lipolysis in adipocytes, but only at submaximal stimulation of lipolysis, *i.e.* close to the physiological levels of noradrenaline. Recently the effect of acetate on the  $^{32}\text{P}$ -phosphorylation of HSL has been studied under the conditions used in paper VI. When 20 mM acetate was added to cells stimulated with 40 nM noradrenaline FFA release was inhibited by 35% but there was no change in the HSL phosphorylation (relative values obtained by scanning densitometry was  $62.3 \pm 1.4$  (8) for acetate and  $61.3 \pm 1.2$  (9) for the parallel control (mean  $\pm$  SEM (n determinations))). Interestingly, this is the only case when inhibition of lipolysis does not seem to have been accompanied by a decrease in the HSL phosphorylation state. Other short-chain carboxylic acids, propionate and  $\beta$ -hydroxybutyrate, can be shown to inhibit adrenaline-stimulated lipolysis in isolated adipocytes from reindeers (T. Larsen and N.Ö. Nilsson, unpublished work). Food digestion in ruminants results in production of propionate, acetate and  $\beta$ -hydroxybutyrate. The studies suggest that these energy substrates cause build-up of depot fat by inhibiting lipolysis (T. Larsen, personal communication).

# THE ANTILIPOLYTIC EFFECT OF INSULIN - HYPOTHETICAL MECHANISM(S) OF ACTION

The finding that insulin prevented or inhibited noradrenaline-stimulation of lipolysis by a corresponding prevention or inhibition of the HSL phosphorylation caused by this hormone has been presented above. The underlying mechanism for this insulin effect cannot be decided from the data of this investigation, but an attempt will be made to relate it to some recently presented hypothetical mechanisms for the short-term action of insulin, and to examine to what extent these can explain the insulin effect on HSL. Naturally, this will represent a partial and, in many ways, speculative view on the subject.

During recent years it has become evident that a large number of short-term insulin actions are mediated by effects on the phosphorylation state of various key regulatory enzymes or enzyme inhibitors (Table 1). Also the insulin effects on glucose transport have been suggested to be related to phosphorylation of certain membrane proteins (Chang and Cuatrecasas, 1974). However, the mode of insulin action on the phosphorylation state varies. The most common effect (Table 1, group 1) is that described for HSL: catecholamines increase and insulin decreases the extent of phosphorylation and the hormones have antagonistic effects on enzyme activity/biological function. In two cases, the high affinity (low  $K_m$ ) phosphodiesterase (Table 1, group 2) and acetyl-CoA carboxylase (Table 1, group 3), recent data (Marchmont and Houslay, 1980; Brownsey *et al.*, 1981) suggest that both catecholamines and insulin increase the phosphorylation state, with synergistic and antagonistic effects on the enzyme activity, respectively. In the case of ATP-citrate lyase (Table 1, group 4) the same is true concerning the effects on the phosphorylation state (Alexander *et al.*, 1979), but so far no well-documented change of the enzyme activity has been demonstrated. Some of these diverse effects of insulin can only be explained in terms of selective phosphorylation by insulin of specific sites and this has also been found in one case, the acetyl-CoA carboxylase (Brownsey *et al.*, 1981, and personal communication).

**Table 1**  
Summary of effects of catecholamines and insulin on the extent of phosphorylation and activity of some important enzymes.

| Group | Enzymes                                       | Ref. | Effect on phosphorylation state by |           | Activity<br>(catecholamine<br>versus insulin) |
|-------|-----------------------------------------------|------|------------------------------------|-----------|-----------------------------------------------|
|       |                                               |      | catecholamines<br>and/or glucagon  | insulin   |                                               |
| 1     | Hormone-sensitive lipase                      | a    | increase*                          | decrease* | opposite                                      |
|       | Pyruvate kinase                               | b    | "-"                                | "-"       |                                               |
|       | Glycogen synthase                             | c    | "-"                                | "-"       |                                               |
|       | Inhibitor 1 (protein<br>phosphatase modifier) | d    | "-"                                | "-"       |                                               |
|       | Phosphorylase kinase                          | e    | "-"                                | "-"       |                                               |
|       | HMG-CoA reductase and its<br>kinase           | f    | "-"                                | "-"       |                                               |
|       | Phosphofructokinase                           | g    | "-"                                | "-"       |                                               |
|       | Pyruvate dehydrogenase                        | h    | "-"                                | "-"       |                                               |
| 2     | Low $K_m$ phosphodiesterase                   | i    | increase                           | increase  | the same                                      |
| 3     | Acetyl-CoA carboxylase                        | j    | increase*                          | increase* | opposite                                      |
| 4     | ATP-citrate lyase                             | k    | increase*                          | increase* | no change                                     |

\*This hormonal effect on phosphorylation state has been directly demonstrated in intact cells or *in vivo*.

- |                                    |                                 |
|------------------------------------|---------------------------------|
| a) Nilsson <i>et al.</i> 1980 (=V) | b) Engström 1980                |
| c) Mc Cullough and Walsh 1979a     | d) Foulkes <i>et al.</i> 1980   |
| e) Mc Cullough and Walsh 1979b     | f) Ingebritsen and Gibson 1980  |
| g) Hofer and Sorensen-Ziganke 1979 | h) Hughes <i>et al.</i> 1980    |
| i) Marchmont and Houslay 1980      | j) Brownsey <i>et al.</i> 1981  |
|                                    | k) Alexander <i>et al.</i> 1979 |

From a discussion with Roger Brownsey, Bristol; Philip Cohen, Dundee; Lorenz Engström, Uppsala; Leonard Jarett, Philadelphia; Hugh Nimmo, Glasgow; Örjan Zetterqvist, Uppsala, and others at the lecture course "The mechanism of action of peptide hormones and catecholamines", Lund, April 22, 1981.

Insulin changes HSL activity by decreasing its phosphorylation state (Table 1). The question can then be asked how this insulin effect is mediated. Obviously, separate mechanisms may operate for the various enzymes of group 1 in Table 1 since different protein kinases may catalyze their phosphorylation, HSL probably being phosphorylated by cyclic AMP-dependent protein kinase, as discussed above. On the other hand many of these enzymes may be dephosphorylated by a single protein phosphatase, protein phosphatase-1 (Cohen, 1980; Ingebritsen *et al.*, 1980). This is true for glycogen synthase, HMG-CoA reductase, HMG-CoA reductase kinase, protein phosphatase inhibitor-1 and may also be the case for HSL (H. Olsson, P. Strålfors and P. Belfrage, unpublished work). Thus, for several of the enzymes of group 1, including HSL, the mechanism through which insulin mediates their net dephosphorylation may involve inhibition of cyclic AMP-dependent protein kinase and/or activation of protein phosphatase 1.

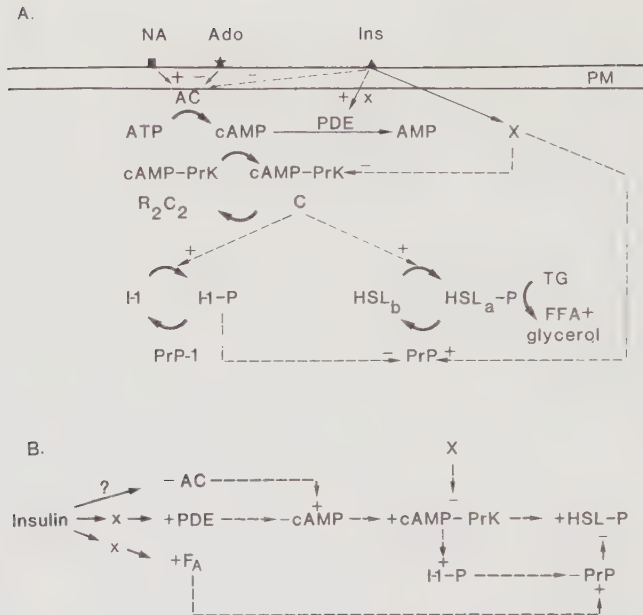


Fig. 7. A. Hypothetical model for the regulation of lipolysis in adipocytes by noradrenaline and insulin.

B. Postulated mechanisms for the decrease of the phosphorylation state of hormone-sensitive lipase by insulin.

PDE indicates cyclic AMP phosphodiesterase; I-1, protein phosphatase inhibitor-1; I-1-P, the phosphorylated form of I-1; PrP-1, protein phosphatase-1;  $x$ , chemical mediator(s) of insulin action;  $F_A$ , activator protein of ATP-Mg-dependent protein phosphatase; dashed lines indicate stimulation (+) or inhibition (-); all other symbols have been identified in Fig. 3.

In Fig. 7A and B a number of recently postulated mechanisms for short-term insulin actions have been used to construct a hypothetical model which could account for the various features of the anti-lipolytic action of insulin described in the present investigation.

One important characteristic of the anti-lipolytic effect was the reduction of the noradrenaline-induced elevation of cyclic AMP (VII). This reduction of cyclic AMP, a consistent finding in intact adipose tissue and in isolated fat cells, at least when cyclic AMP levels are moderately elevated (Butcher *et al.*, 1968, Manganiello and Vaughan, 1973; Kono and Barham, 1973; Soderling *et al.*, 1973; Knight and Iliffe, 1973), could lead to a decrease of the HSL phosphorylation state in two ways (Fig.

7A and B). Firstly, it could decrease HSL phosphorylation through decreased cyclic AMP-dependent protein kinase activity. Secondly, amplifying this effect, it could increase HSL dephosphorylation through increased protein phosphatase activity. This assumes that protein phosphatase-1 accounts for the dephosphorylation of HSL, that this phosphatase is regulated by inhibitor-1 (Cohen, 1980) and that the phosphorylation state and activity of inhibitor-1 is decreased through the inhibition of the cyclic AMP-dependent protein kinase (Cohen, 1980).

The lack of insulin effect on maximally dibutyryl cyclic AMP-stimulated HSL phosphorylation and lipolysis (VI) is consistent with this postulated cyclic AMP-dependent mechanism for the anti-lipolytic action of insulin. Insulin could have reduced cyclic AMP through activation of low  $K_m$  phosphodiesterase (Lotten and Sneyd, 1970) via phosphorylation of this enzyme (Marchmont and Houslay, 1980), or possibly through inhibition of adenylate cyclase (Hepp, 1972; Lambert and Jacquemin, 1979) (Fig. 7A,B). The insulin effects on the phosphodiesterase may have been mediated through the low molecular weight insulin mediators (Jarett *et al.*, 1981) (further discussed below).

Although reduction of cyclic AMP has been implied as a mechanism for the anti-lipolytic action of insulin for a long time, it has been hard to reconcile with the fact that in many cases cyclic AMP levels correlate poorly quantitatively with the effects on lipolysis (Fain, 1980), and with the HSL phosphorylation state (VII). One possible explanation to the lack of correlation is compartmentalization of cellular cyclic AMP, with only a small functional pool of cyclic AMP directly responding to hormonal perturbation. Evidence for such compartmentalization has indeed been provided recently by several workers (Corbin *et al.*, 1977, Hayes *et al.*, 1979, Hayes *et al.*, 1980) and could be used as an argument for cyclic AMP reduction as the only mechanism for the anti-lipolytic effect of insulin. On the other hand cyclic AMP compartmentalization could also be used as an argument to invalidate any observation of a good correlation between measured cyclic AMP concentration and insulin effects on lipolysis.



In view of the insulin effects on enzyme systems apparently regulated by cyclic AMP-independent mechanisms *e.g.* HMG-CoA reductase (Ingebritsen and Gibson, 1980) and pyruvate dehydrogenase (Denton *et al.*, 1975) it still seems likely that part of the insulin decrease of the HSL phosphorylation state (VII) would be due to cyclic AMP-independent mechanism(s). Such effects (Fig. 7A,B) could have been caused through the low molecular weight chemical mediators described by Larner *et al.*, (1979) and Jarett and Seals (1979), by inhibition of cyclic AMP-dependent protein kinase (Larner *et al.*, 1979) and/or by activation of protein phosphatase (Jarett and Seals, 1979). The activation may possibly involve the transformation of an inactive to an active protein phosphatase through its phosphorylation (Cohen, 1980). The chemical mediators would have to activate the protein phosphatase through an intermediary amplifying step, perhaps by acting on a protein kinase, in much the same way as cyclic AMP activates cyclic AMP-dependent protein kinase. The  $F_A$  factor described by Goris *et al.* (1979) which seems to activate a multifunctional protein phosphatase (Goris *et al.*, 1979), possibly identical with an inactive form of protein phosphatase-1 (Cohen, 1980), has been suggested to be a protein kinase (Cohen, 1980). An interesting possibility would be that this  $F_A$  component would be the target protein kinase for one of the postulated insulin chemical mediators.

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# Effects of acetate, acetaldehyde, and ethanol on lipolysis in isolated rat adipocytes

Nils Östen Nilsson and Per Belfrage

Department of Physiological Chemistry, University of Lund, Lund, Sweden

**Abstract** The effects of different concentrations of acetate, acetaldehyde, and ethanol, alone or in combination, on the lipolysis rate, measured as glycerol release, were studied in isolated adipocytes from fed or fasted rats, in the basal state and at various levels of norepinephrine stimulation. Acetate inhibited the glycerol release in a dose-dependent manner ( $\approx 10\%$  inhibition at 2 mM, 25–70% at 10 mM) with the most marked effects at low to moderate norepinephrine concentrations (1.5–7 ng/ml). Little or no inhibition was observed in the absence of hormone and at maximal (100 ng/ml) hormone stimulation. Ethanol, up to 100 mM concentration, had no effect on the lipolysis rate. Acetaldehyde, up to 1 mM concentration, had no reproducible effect. Ethanol, acetaldehyde, and acetate in combination inhibited glycerol release to an extent similar to that of acetate alone.

**Supplementary key words** glycerol · spectrophotofluorometry · norepinephrine

The intake of small or moderate amounts of ethanol by man (1–4) or rats (5) is known to be rapidly followed by a decrease of plasma glycerol and free fatty acid concentrations. Since ethanol intake does not increase the uptake and utilization of glycerol (6, 7), this decrease must be the result of a lowered glycerol production, i.e., lipolysis rate in the adipose tissue.

The cause for the diminished lipolysis rate in adipose tissue has not been established. Intake of small or moderate amounts of ethanol has had no marked effects on the levels of lipolytic (8) or antilipolytic hormones (9). Infusion of acetate, the major metabolite of ethanol oxidation, into man decreased plasma FFA (10). It has therefore been suggested that acetate is responsible for the antilipolytic action of ethanol intake (11). However, *in vitro* experiments have given conflicting results. Both ethanol and acetaldehyde have been shown to have no influence or to have either stimulatory or inhibitory effects on lipolysis, depending on concentrations and other experimental conditions (12–14). Acetate slightly inhibited basal lipolysis in isolated adipocytes (15).

These divergent results prompted us to perform a

more systematic study of the effects of ethanol and its oxidation metabolites, acetaldehyde and acetate, on the lipolysis rate in adipose tissue. Glycerol release, in the basal state and at various stages of stimulation with the lipolytic hormone norepinephrine, has been used as a measure of this parameter. The isolated adipocytes from epididymal fat pads of fed or fasted rats were employed as a model system.

## MATERIALS AND METHODS

Isolated fat cells were prepared by the collagenase digestion method of Rodbell (16) as modified by Gliemann (17) from epididymal fat pads and perirenal fat of 120–140 g male Sprague-Dawley rats (Anticimex, Stockholm, Sweden). The animals were either given laboratory chow (R3, Astra-Evos, Södertälje, Sweden) *ad libitum* or fasted overnight. The cells were suspended in Krebs-Ringer-HEPES buffer pH 7.4 (NaCl, 118.7 mM; KCl, 4.75 mM; CaCl<sub>2</sub>, 2.54 mM; KH<sub>2</sub>PO<sub>4</sub>, 1.19 mM; MgSO<sub>4</sub>, 1.19 mM; HEPES, 24.6 mM) containing 3.5% (w/v) bovine serum albumin and 2.5 mM glucose. Cell concentration was measured as packed cell volume (PCV) after centrifugation in microhematocrit tubes (18). One ml of PCV from fed rats corresponded approximately to 0.7 g of extracted lipids and 40 mg of protein. Hormone sensitivity of the cells was routinely checked by determining the insulin stimulation of [<sup>3</sup>H]glucose uptake and conversion into lipids (19). Only cells showing a 15–30-fold maximal insulin stimulation of this parameter were used (cf. ref. 19).

One ml portions (10–15  $\mu$ l PCV) of a pooled cell suspension were transferred to polyethylene counting vials for incubation. After preincubation for 15 min at 37°C, zero-time samples were taken and norepineph-

Abbreviations: FFA, free fatty acids; HEPES, 2-[4-(2-hydroxyethyl)-piperazinyl-(1)]-ethane-sulfonic acid; PCV, packed cell volume.

rine or other substances (20–50  $\mu$ l, aqueous solution) were added. Incubations were performed in a metabolic shaker (80 cycles/min, stroke length 4 cm) at 37°C with air as gas phase for the desired time (60 min, unless otherwise stated). They were terminated by quickly separating fat cells from medium by floating them through a silicone oil layer (18) in a polypropylene centrifuge tube (1 min, 10,000 *g*, Beckman Microfuge B). After cutting the tube just below the oil layer, a portion of the medium was taken for glycerol analysis.

Glycerol analysis was performed with an enzymatic fluorometric method (20) modified to obtain higher sensitivity. Two hundred  $\mu$ l of incubation medium or glycerol standard solution (0–200  $\mu$ M) was mixed with 200  $\mu$ l each of  $\text{ZnSO}_4$  (87 mM) and  $\text{Ba(OH)}_2$  (83 mM) and the precipitated proteins were removed by centrifugation. A 200  $\mu$ l portion of the clear supernatant was then incubated for 75 min at room temperature with 300  $\mu$ l of enzyme reagent mixture (8.7 ml of hydrazinium-HCl, 0.33 M, pH 9 with  $\text{MgCl}_2$ , 1.5 mM; 1.0 ml of cystein, 35 mg/ml; 0.1 ml of EDTA, 0.1 M, pH 9; 0.2 ml of ATP, 20 mM; 0.2 ml of NAD, 30 mM; 1  $\mu$ l

of glycerol kinase; and 10  $\mu$ l of glycerol phosphate dehydrogenase) and 500  $\mu$ l of 10 mM NaOH with 0.5 mM EDTA added. The fluorescence ( $\lambda_{\text{exl}}$  340 and  $\lambda_{\text{eml}}$  455 nm) was then determined. The glycerol standard curve was linear up to at least 100  $\mu$ M and fluorescence was within 10% of expected NADH values. Glycerol release was calculated as the difference between the samples at the end of the incubation and the zero-time values and was expressed as  $\mu$ mol/ml PCV per hr. The statistical comparison of control and experimental samples was made with Student's *t* test, comparison of means of two independent samples. Reproducibility within the same cell batch was 10% for basal and 2% for maximally stimulated lipolysis, and sensitivity ( $2 \times \text{SD}$  of zero-time samples) was 0.3 nmol of glycerol, corresponding to 0.1  $\mu$ mol/ml PCV.

Norepinephrine bitartrate was from Apoteksbolaget, Stockholm, Sweden and acetaldehyde (99% pure) was from BDH Chemicals; bovine serum albumin (Cohn's fraction V) and NAD (grade V) were from Sigma Chem. Co, St. Louis, MO. Glycerokinase (E.C. 2.7.1.30), 1 mg/ml; glycerol-3-phosphate dehydrogenase (E.C.

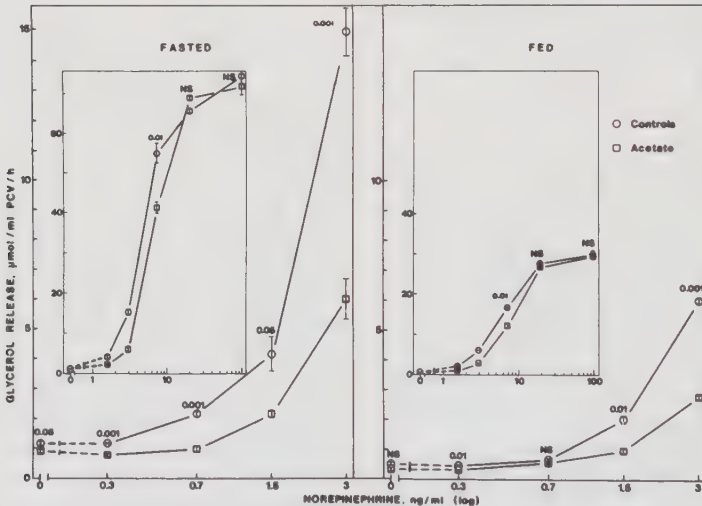


Fig. 1. Acetate inhibition of glycerol release at different norepinephrine concentrations. Adipocytes (12  $\mu$ l PCV/ml) from fasted or fed rats were incubated (after 15 min preincubation) for 60 min with and without acetate (10 mM) at the indicated concentrations of norepinephrine in Krebs-Ringer-HEPES buffer, pH 7.4, with 3.5% albumin and 2.5 mM glucose at 37°C. The vials were shaken 80 cycles/min, stroke length 4 cm. Glycerol released to the medium was measured enzymatically-fluorometrically and related to the amount of adipocytes expressed as ml packed cell volume (PCV). Values are mean  $\pm$  SEM,  $n = 3$  (number of samples from one cell batch), and the statistical significance was as indicated in the figure. N.S. signifies nonsignificant ( $P > 0.05$ ).

1.1.1.8), 10 mg/ml; and ATP were from Biochimica Boehringer, Mannheim. Hydrazinium hydrochloride (24%) was from Merck, Darmstadt, and HEPES was from Schwarz-Mann.

The bovine serum albumin was extensively dialyzed against distilled water and filtered through a 0.8- $\mu$ m Millipore filter before use. The fatty acid content as determined by gas-liquid chromatography was approximately 0.25 mol per mol of albumin. Serum was prepared from rats fasted overnight.

## RESULTS

### Effects of acetate on glycerol release

Acetate (10 mM) inhibited glycerol release at various stages of norepinephrine stimulation with fat cells from both fed and fasted rats (Fig. 1). Little or no effect on basal or maximally hormone-stimulated glycerol release occurred, while inhibition was prominent (25–70%) at low to moderate norepinephrine concentrations. This pattern of inhibition by acetate was similar with cells from both fed and fasted rats, but there was a tendency for less effect with fat cells from fed rats at the lowest norepinephrine concentrations (Fig. 1). The acetate inhibition of moderately norepinephrine-stimulated glycerol release was constant with incubation time, possibly excepting the first few minutes (Fig. 2). It was clearly dependent on acetate concentration, roughly linear with the logarithm of this parameter, and with no difference due to nutritional state (Fig. 3). Statistically significant inhibition

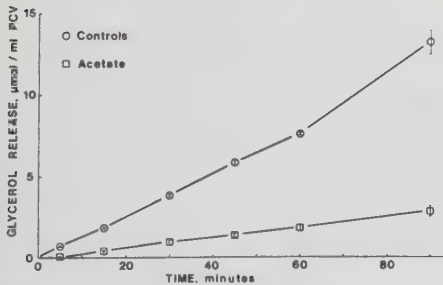


Fig. 2. Time course of acetate inhibition of norepinephrine-stimulated glycerol release. Adipocytes (15  $\mu$ l PCV/ml) from fed rats were incubated during the indicated time with and without acetate (20 mM) and with norepinephrine (3 ng/ml) under the conditions described in Fig. 1. Values are mean  $\pm$  SEM,  $n = 3$ . All values except those at the two shortest time intervals are statistically significant ( $P < 0.01$ ).

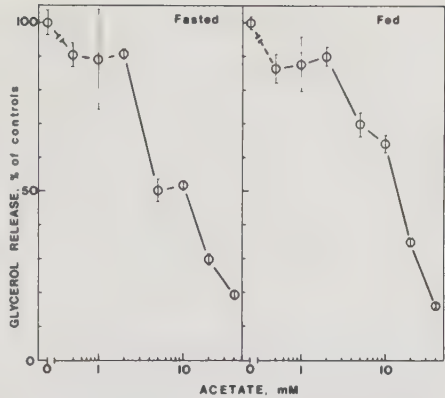


Fig. 3. Effects of varying acetate concentration on norepinephrine-stimulated glycerol release. Adipocytes from fasted and fed rats (15  $\mu$ l PCV/ml) were incubated for 60 min without and with the indicated concentrations of acetate and with norepinephrine (3 ng/ml) under the conditions described in Fig. 1. Values are mean  $\pm$  SEM,  $n = 4$ . Inhibition was statistically significant ( $P < 0.05$ ) from 2 mM acetate concentration.

( $P < 0.05$ ) could often be demonstrated at 1–2 mM acetate concentration.

### Effects of ethanol

Ethanol (25 mM) had no statistically significant effect on glycerol release from fat cells from fed (Fig. 4) or fasted rats (data not presented), in the absence or presence of the indicated concentrations of norepinephrine. Furthermore, no effect could be observed with ethanol concentrations up to 100 mM at a moderately hormone-stimulated glycerol release rate (3 ng/ml of norepinephrine) (Table 1), while the high concentration of 400 mM (approx. 2% v/v) resulted in a three-fold increase of basal glycerol release but had little effect on the maximally hormone-stimulated rate. The reported ethanol inhibition of serum-stimulated glycerol release (14) was tested with 25 mM ethanol and serum diluted (1:1 v/v) with the incubation buffer. A slight (average 25%) but statistically significant ( $P < 0.001$ ) decrease was obtained with cells from fasted rats (data not presented).

### Effects of acetaldehyde and combined effects

Acetaldehyde (1–1000  $\mu$ M) had no reproducible effect on moderately (3 ng/ml) norepinephrine-stimulated glycerol release in any nutritional state. Acetaldehyde (100  $\mu$ M) had no reproducible effect on

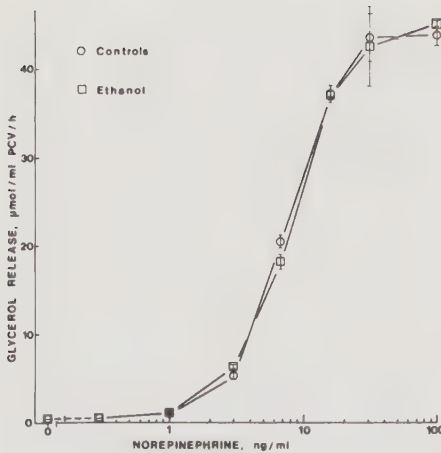


Fig. 4. Lack of effect of ethanol on glycerol release at different concentrations of norepinephrine. Adipocytes (15  $\mu$ l PCV/ml) from fed rats were incubated with and without ethanol (25 mM) at the indicated concentrations of norepinephrine under the conditions described in Fig. 1. Values are mean  $\pm$  SEM,  $n = 3$ . The difference between ethanol and control was in no case statistically significant ( $P > 0.05$ ).

glycerol release at any stage of norepinephrine stimulation (1–100 ng/ml).

The inhibition of glycerol release by acetate (10 mM), ethanol (25 mM), and acetaldehyde (100  $\mu$ M) in combination was similar to that of acetate (10 mM) alone (Table 2).

## DISCUSSION

The high sensitivity of the modified enzymatic, fluorometric method for glycerol determination made

TABLE 1. Lack of effect of ethanol on glycerol release from isolated fat cells

| Ethanol | Glycerol Release    |
|---------|---------------------|
| mM      | $\mu$ mol/ml PCV/hr |
| 0       | 14.4 $\pm$ 0.54     |
| 0.01    | 13.3 $\pm$ 0.55     |
| 0.1     | 14.5 $\pm$ 0.34     |
| 1       | 15.3 $\pm$ 0.63     |
| 10      | 16.6 $\pm$ 1.1      |
| 25      | 15.4 $\pm$ 0.30     |
| 100     | 14.6 $\pm$ 0.74     |

Adipocytes (9.7  $\mu$ l PCV/ml) from fed rats were incubated without and with ethanol at the indicated concentrations in the presence of norepinephrine (3 ng/ml) for 60 min under the conditions described in Fig. 1. Values are mean  $\pm$  SEM,  $n = 5$ .

TABLE 2. Effects of ethanol, acetaldehyde, and acetate alone or in combination on glycerol release from isolated fat cells

| Addition                                                  | Glycerol Release | P      |
|-----------------------------------------------------------|------------------|--------|
|                                                           | % control        |        |
| None                                                      | 100              |        |
| Ethanol, 25 mM                                            | 103.1 $\pm$ 6.9  | N.S.   |
| Acetaldehyde, 0.1 mM                                      | 99.4 $\pm$ 4.3   | N.S.   |
| Acetate, 10 mM                                            | 56.9 $\pm$ 6.1   | <0.001 |
| Ethanol + acetaldehyde + acetate, concentrations as above | 62.6 $\pm$ 5.2   | <0.001 |

Adipocytes representing seven separate cell batches from fasted rats were incubated with the additions stated above at the indicated concentrations and in the presence of norepinephrine (3 ng/ml) for 60 min under the conditions described in Fig. 1. Values are mean  $\pm$  SEM of the values obtained in the seven different experiments, each based on 3–5 incubations.

it possible to use as little as 10–15  $\mu$ l PCV of fat cells in each incubation. This was of considerable practical value when a large number of incubations had to be performed with the same cell batch, e.g., in the experiments with the different norepinephrine concentrations described in Fig. 1.

The most significant finding of the present work was the consistent inhibition of glycerol release from adipocytes by acetate. This inhibition was observed at acetate concentrations comparable with those obtained in the circulating blood after ethanol ingestion (21) and at low to moderate hormone stimulation of lipolysis. These facts and the observation by others of a decrease of plasma free fatty acid concentration after acetate infusion into man (10) in combination with the lack of effects of ethanol and acetaldehyde on the lipolysis rate in this work indeed suggest that acetate is responsible for the antilipolytic effects of ingestion of small or moderate amounts of ethanol (1–5). The stimulatory effects of ethanol at 400 mM concentration reported by others (12) and also found by us is of little relevance in this connection, in view of the much lower concentrations obtained after ethanol ingestion. The same is true for the stimulatory effects of acetaldehyde at 1 mM concentration described by others in view of arterial blood levels of 20–160  $\mu$ M (22) reported after ethanol ingestion. Lactate, which is produced at a higher than normal rate during ethanol metabolism (23) and which could theoretically influence the lipolysis in adipose tissue, has recently been shown to have no effect on this parameter in isolated fat cells (24).

The present work does not provide any evidence for the mechanism of the acetate effect. Acetate is known to be metabolized by adipose tissue (15). Most (50%) of the acetate produced during ethanol oxidation is believed to be oxidized by skeletal muscle (25)

but it is possible that a considerable part of the remainder may be disposed of by the adipose tissue. If so, acetate may have several effects on the intermediary metabolism of this tissue, which could possibly effect the rate of lipolysis. Alternatively, acetate, like several short-chain carboxylic acids and  $\beta$ -hydroxybutyrate (24), might inhibit adenosine 3':5'-monophosphate (cyclic AMP) accumulation, e.g., by interference with adenyl cyclase. 

Mrs. Ingrid Nordh gave skillful technical assistance. Grants were obtained from the Medical Faculty, University of Lund, and the Swedish Medical Research Council (Project No. 3362).

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## notes on methodology

### Continuous monitoring of free fatty acid release from adipocytes by pH-stat titration

Nils Östen Nilsson and Per Belfrage

Department of Physiological Chemistry 4, University of Lund, P.O.B. 750, S-220 07 Lund, Sweden

**Summary** A method for direct and continuous monitoring of free fatty acid release in adipocyte suspensions is described. Using a pH-stat apparatus the protons from the released free fatty acids are continuously titrated and the accumulated amount of  $\text{OH}^-$  added is monitored on a recorder against time, the slope thus indicating the rate of free fatty acid release. Since pH is kept constant, an incubation medium with a low buffering capacity can be used, which gives the method a high sensitivity. Under the conditions described, free fatty acid release from 5% of maximal norepinephrine stimulation of rat adipocytes can be accurately measured and the kinetics can be followed over extended periods of time. — Nilsson, N. Ö., and P. Belfrage. Continuous monitoring of free fatty acid release from adipocytes by pH-stat titration. 1979. *J. Lipid Res.* 20: 557–560.

**Supplementary key words** lipolysis · buffer capacity · norepinephrine · insulin

Release of free fatty acids (FFA) from adipose tissue slices is associated with an equivalent production of protons leading to a decline of the pH of the incubation medium (1). With isolated rat adipocytes that have been stimulated with lipolytic hormones, the pH decrease can be monitored and the rate of FFA release calculated (2). However, the pH decrease in itself influences this rate (3). Furthermore, changes in the concentration of albumin, used as a FFA acceptor, or in  $\text{K}^+$  concentration influence lipolysis differently at reduced and normal pH levels (3). In this note we describe a method for the determination of FFA release from adipocytes in suspension under such conditions that the medium pH is kept constant and the difficulties above are thereby avoided. The FFA release is monitored continuously by titration of the stoichiometrical amount of protons released, with the use of a recording pH-stat titration apparatus.

#### PROCEDURE

##### Preparation of adipocytes

Rat adipocytes were prepared according to Rodbell (4) as modified by Gliemann (5) from epididymal and

perirenal fat from 120–150 g male Sprague–Dawley rats (Anticimex, Stockholm, Sweden) using 0.5 mg/ml of collagenase in Krebs–Ringer buffer with HEPES, 24 mM; albumin, 3.5% (w/v); and glucose, 0.55 mM. The rats were fed a standard pellet diet. The adipocyte concentration was determined (6) as packed cell volume (PCV) and the cell suspension was diluted to 50  $\mu\text{l}$  PCV/ml in Krebs–Ringer buffer with HEPES, 24 mM; albumin, 1% (w/v); and glucose, 0.55 mM (storage medium).

The cell number in 1  $\mu\text{l}$  of PCV was calculated from the cell diameter, measured with an ocular micrometer; the small volume of medium trapped between the cells, about 1% (6), was neglected in the calculation. Since the cell number in 1  $\mu\text{l}$  of PCV is greatly affected by the cell diameter, and cell size increases with the weight of the rats, it is important to use rats of the same weight to minimize day to day variation. One  $\mu\text{l}$  of PCV contained  $1.58 \cdot 10^4 \pm 0.23 \cdot 10^4$  cells from rats of weight  $137 \pm 12.6$  g (mean  $\pm$  SD,  $n = 10$ ).

Viability was tested by microscopic appearance and by insulin stimulation of the conversion of medium [ $3\text{-}^3\text{H}$ ]glucose into adipocyte  $^3\text{H}$ -labeled lipids (7). Only cells that gave at least a 10-fold maximal increase of this parameter were used. Half maximal stimulation usually occurred at 7  $\mu\text{U/ml}$  of insulin.

Human adipocytes were prepared from surgical biopsy specimens taken during general anesthesia. The biopsy specimens were minced in the collagenase solution immediately after removal and treated in the same way as the rat tissue. Viability was tested by microscopic appearance. The cell number per  $\mu\text{l}$  of PCV was  $6.3 \cdot 10^4$  for the experiment described in Fig. 4.

##### Incubation conditions and pH-stat titration

Aliquots of 2 ml of cell suspension (50  $\mu\text{l}$  PCV/ml) were stored for up to 4 hr in polyethylene vials at 37°C in a water bath with gentle shaking. Immediately before starting each pH-stat titration the storage medium was removed and replaced with (unless otherwise stated) a modified Krebs–Ringer buffer with low buffering capacity: NaCl, 131 mM; KCl, 4.95 mM;  $\text{CaCl}_2$ , 2.54 mM;  $\text{KH}_2\text{PO}_4$ , 1.19 mM;  $\text{MgSO}_4$ , 1.19 mM; bovine serum albumin, 1% (w/v); pH 7.40 (incubation medium). For this replacement the cell suspension (100  $\mu\text{l}$  PCV of cells) was transferred to a centrifuge tube containing a thin polyethylene tubing.

Abbreviations: FFA, free fatty acids; HEPES, 2[4-(2-hydroxyethyl)-piperazinyl-(1)]-ethanesulfonic acid; PCV, packed cell volume; NE, norepinephrine; Ins, insulin.

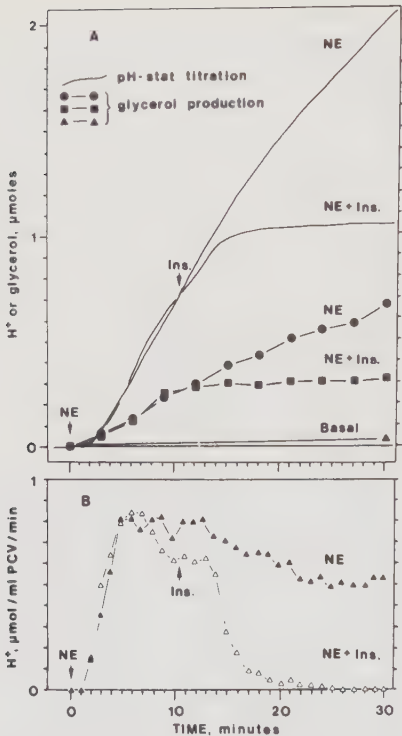


Fig. 1. A. Recorder charts from pH-stat titration experiments. Proton release from suspended rat adipocytes (10  $\mu$ l PCV/ml) was measured by continuous titration under basal conditions, after the addition of norepinephrine (final concentration 5 ng/ml), or norepinephrine followed by insulin (10  $\mu$ U/ml). Glycerol concentration was measured enzymatically fluorimetrically in single samples (150  $\mu$ l) from the incubation mixture and plotted in the same scale. Each set of data are from one representative experiment. No correction has been made for the volume change during the incubation. B. Rates of proton release calculated over 1-min intervals from the pH-stat data of the experiments in Fig. 1A. The data have been corrected for the volume change caused by the glycerol sampling.

After centrifugation (100  $g$  for 30 sec at room temperature) the storage medium under the floated cells was removed through the thin tubing and the cells were resuspended in fresh incubation medium, usually 10 ml (37°C). The low cell concentration (10  $\mu$ l PCV/ml) was chosen to keep the hormonal degradation low (8). The pH-stat incubation vial (polystyrene, 25 mm i.d.) was thermostated to  $37 \pm 0.1^\circ\text{C}$  by a water jacket and supplied with a stream of water-saturated

O<sub>2</sub> above the cell suspension. The latter was mixed with a magnetic stirrer set at a speed which just prevented flotation of cells.

The pH-stat titrator was a combination of DK 10, electrode potential amplifier; DK 11, rate and end-point control; DV 11, burette drive; DV 201, 1-ml interchangeable burette; and DV 13/131 automatic valve control, from Mettler Instrumente AG, Greifensee-Zürich, Switzerland. To the pH-stat was fitted a combined glass electrode (EA 120, Metrohm AG, Herisau, Switzerland). Volumes of 0.2  $\mu$ l could be delivered, i.e., 2 nmol of OH<sup>-</sup> with the 10 mM NaOH usually employed. The pH-stat was adjusted to the pH of the incubation medium. After 5–10 min of preincubation (from the change to incubation medium), steady-state conditions were achieved. Then, zero time was usually defined by the addition of norepinephrine. The titrated volume was recorded on a Vitatron 2001 recorder equipped with a Multirange Module B for variation of y-scale amplification. This module was set to 100 mV, and paper movement (x scale) was set to 10 mm/min. The electrode was treated with pepsin (5% in 0.1 M HCl) after each experimental day to maintain its sensitivity.

#### Glycerol and fatty acid determination

Samples (150  $\mu$ l) were taken from 10-ml incubations at various time intervals. The incubation was interrupted by a rapid centrifugation (Beckman Micro-

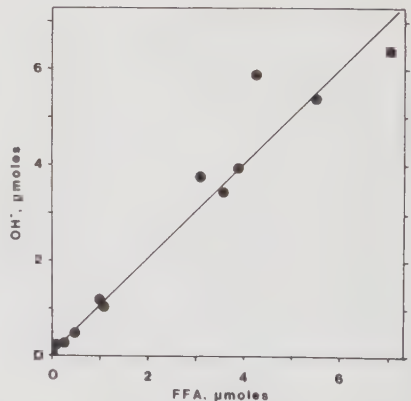


Fig. 2. Relationship between proton and FFA release. Adipocytes were incubated as in Fig. 1 but were stimulated with 100 ng/ml of norepinephrine. Samples of the incubation medium were taken at various times and analyzed for fatty acids by a colorimetric method. Values corrected for medium FFA before hormonal stimulation. Curve calculated by linear regression analysis.  $r = 0.977$ .

fuge B, 1 min, 10,000 g) through a silicone oil layer (6), leaving the medium separated from the cells for analysis. Glycerol was determined after deproteinization with the enzymatic, fluorometric method (9) modified for higher sensitivity (10). FFA was determined as Cu-complexes with a colorimetric method (11).

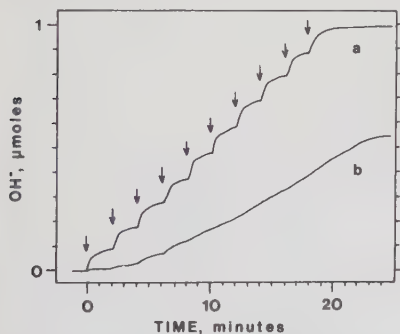
## Materials

Norepinephrine bitartrate was from Sigma Chemical Co., St. Louis, MO; monocomponent porcine insulin was a gift from Novo, Copenhagen, Denmark; [ $^3\text{H}$ ]glucose was from the Radiochemical Centre, Amersham, England; and HEPES (2-[4-(2-hydroxyethyl)-piperazinyl-(1)]-ethanesulfonic acid) was from Schwarz-Mann, NY. Bovine serum albumin (Cohn Fraction V) from Sigma Chemical Co. was extensively dialyzed and filtered (0.8  $\mu\text{m}$ ). Collagenase type CLS (Batch No. 45K137) was from Worthington Biochemical Corp., Freehold, NJ. All other chemicals were of analytical grade.

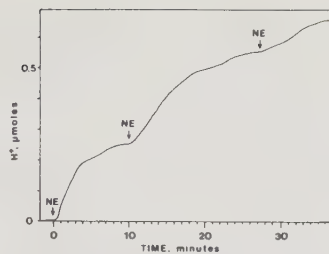
## RESULTS

### FFA release from rat adipocytes continuously monitored by pH-stat titration

The time course of norepinephrine stimulation of FFA production, measured as proton release, and the inhibition of this stimulation by insulin could be conveniently followed by continuous pH-stat titration (Fig. 1A). The effects were even more evident if rates



**Fig. 5.** Effect of buffering capacity of incubation medium on titrations of added fatty acid. One hundred nanomoles of palmitic acid (in 10  $\mu\text{l}$  ethanol) was added to 10 ml of incubation medium *a* or *b* at arrows. *a*, Incubation medium with low buffering capacity used in all cell incubations (see Procedure); *b*, Krebs-Ringer solution buffered with 24 mM HEPES and with 1% (w/v) bovine serum albumin.



**Fig. 4.** FFA release from human adipocytes measured by continuous pH-stat titration. Norepinephrine, 100 ng/ml, added at arrows. Incubation conditions as in Fig. 1. The data are from one representative experiment.

were plotted (Fig. 1B) instead of the cumulative values obtained on the recorder. Under the incubation conditions used, with no glucose in the medium, the FFA release closely paralleled glycerol release, at a molar ratio of 3:1 (Fig. 1A). Thus, in these experiments, the rate of FFA release was also a measure of the rate of lipolysis, and the hormonal effects reflect changes in the activity of the hormone-sensitive lipase of the adipocytes.

The FFA release in Fig. 1 was approximately linear over 15 min. However, the concentration of norepinephrine used (5 ng/ml) gave less than half-maximal stimulation. At maximal stimulation (100 ng/ml) linearity was obtained for 30 min with a lipolytic rate of 2.25  $\mu\text{mol}$  fatty acids released/ml PCV per min from 5 min after norepinephrine addition. Then the FFA/albumin ratio in the incubation medium exceeded 3 and thus was a limiting factor (12). This could be overcome by increasing the albumin concentration. The increase in buffer capacity caused by the increased albumin concentration was of no importance at these high lipolytic rates.

The equivalence between proton and FFA release is demonstrated in Fig. 2. Under the conditions used the FFA release obtained at 5% of maximal norepinephrine stimulation (2 ng/ml) represented the practical lower limit of the method. To obtain this sensitivity it was essential to use an incubation medium with a low buffering capacity (cf. Fig. 3). With the low cell concentration used (10  $\mu\text{l}$  PCV/ml) FFA release in the absence of norepinephrine could not be measured (Fig. 1A).

Adipocytes could be used with the same hormonal sensitivity up to at least 4 hr after preparation if stored at 37°C with gentle shaking. The reproducibility of consecutive titrations was tested using cells from one large cell batch. After stimulation with 5 ng/ml of norepinephrine, the initial rate (calculated from 5–

10 min after norepinephrine addition) was  $0.91 \mu\text{mol/ml PCV per min}$  with a variation coefficient of 5.6% ( $n = 7$ ). The entire experiment lasted for 4 hr. There was no significant difference between the first three and the last three titrations.

The variation coefficient for the lipolytic rate obtained after norepinephrine ( $5 \text{ ng/ml}$ ) stimulation between different cell batches was 12.7% ( $0.89 \pm 0.11$  ( $\bar{X} \pm \text{SD}$ )  $\mu\text{mol FFA/ml PCV per min}$  for seven separate cell batches; the value for each batch was the mean of three titrations).

#### FFA release from human adipocytes

FFA release from human adipocytes could be monitored by continuous pH-stat titration (Fig. 4). However, with human cells the increased FFA release after norepinephrine was linear for only a few minutes, even with amounts of hormone ( $100 \text{ ng/ml}$ ) that gave maximal response in rat adipocytes. Furthermore, the responses after repeated additions of norepinephrine decreased successively (Fig. 4).

The lipolytic rate during the first 5 min was  $0.4 \mu\text{mol FFA/ml PCV per min}$ . It can be calculated to correspond to  $0.5 \mu\text{mol FFA/g triacylglycerols per min}$  ( $80 \text{ nmol}/10^5 \text{ cells per min}$ ). As a comparison, although not directly comparable because of other incubation conditions, lipolytic rates of  $0.2\text{--}0.3 \mu\text{mol FFA/g triacylglycerols per min}$  after incubation of human cells with  $10^{-5} \text{ M}$  isoproterenol were obtained by others (13).

#### DISCUSSION

Continuous pH-stat titration was found to be a convenient and rapid method to monitor FFA efflux from adipocytes, especially when it was desired to follow the time course of effects after perturbation with hormones and other agents. The method can also be used to follow the rate of lipolysis, i.e., the rate-limiting activity of the hormone-sensitive lipase (14), under such conditions that little reesterification of liberated fatty acids take place. Metabolites and enzymatic activities can easily be determined in samples of cells and medium taken at selected time points during the continuous monitoring of hormonal stimulation. The method should thus be of general value in studies of hormonal effects.

FFA release from adipocytes can also be followed as a decline of the pH of the incubation medium (2). However, the pH decrease in itself rapidly changes the conditions for the FFA release (cf. 3), especially when a high sensitivity is required and incubation medium with a low buffering capacity therefore is used. In

contrast, in the present method the protons produced are continuously titrated and the pH is maintained constant regardless of the buffering capacity of the incubation medium. In our work the pH-stat titration technique has proven to be a more versatile instrument, particularly when the kinetics for the FFA release at low concentrations of hormones are studied.

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## [20] Continuous Measurement of Free Fatty Acid Release from Intact Adipocytes by pH-Stat Titration

By NILS ÖSTEN NILSSON and PER BELFRAGE

Free fatty acids (FFA) are released into the incubation medium from intact, suspended adipocytes when these cells are exposed to a variety of lipolytic hormones and drugs. Protons are released into the extracellular medium from the carboxyl groups of FFA, essentially stoichiometrically at pH 7.4.<sup>1</sup> The protons, and thus FFA, can be measured continuously by titration with NaOH at pH 7.4 using an automatic pH-stat titration apparatus.<sup>2</sup> The amount of NaOH registered versus time on a recorder accu-

<sup>1</sup> D. Rudman and P. W. Shank, *Endocrinology* **79**, 1100 (1966).

<sup>2</sup> N. Ö. Nilsson and P. Belfrage, *J. Lipid Res.* **20**, 557 (1979).



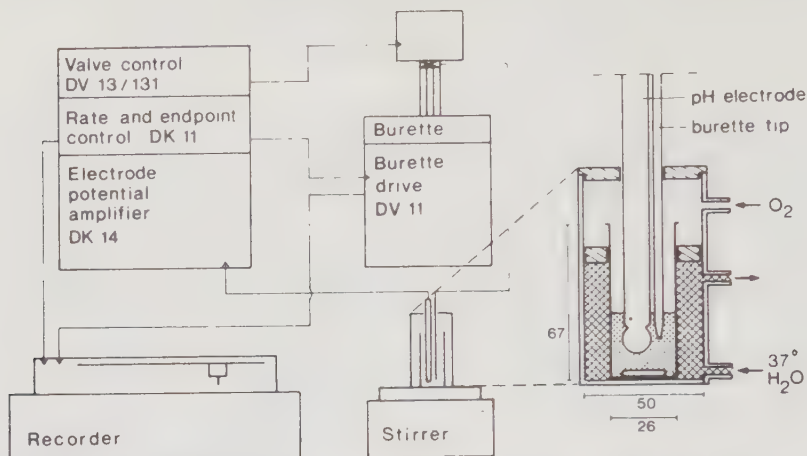


FIG. 1. pH-stat titration equipment for continuous measurement of release of free fatty acids from isolated adipocytes. *Inset*: Magnified view of incubation vial; dimensions are given in millimeters.

rately monitors the accumulation of released FFA in the incubation medium. Release of other acid metabolites, e.g., lactate, does not contribute significantly to the proton release except under conditions of basal (nonstimulated) lipolysis.<sup>3</sup> Rate of FFA release can be registered directly as the first derivative of the output values from the pH-stat titrator. The sensitivity of the method depends on a low buffering capacity of the incubation medium, the limit being set by the minimal concentration of albumin needed for its proper function as fatty acid acceptor.

### Apparatus

It is essential that (a) the pH-stat titrator be sufficiently sensitive to small changes of the electrode potential (corresponding to approximately  $10^{-3}$  pH unit); (b) the titration rate be varied automatically within a wide range to cover marked changes of lipolysis rate. We have found useful an apparatus from Mettler Instrumente AG, Greifensee-Zürich, Switzerland. It consists of the following parts (Fig. 1): electrode potential amplifier (DK 10 or DK 14), rate and end-point control (DK 11), burette drive (DV 11), 1-ml interchangeable burette (DV 201) and automatic valve control (DV 13/131). The volume of titrant is monitored on a recorder (Vitatron 2001,

<sup>3</sup> J. P. Flatt and E. G. Ball, *J. Biol. Chem.* **239**, 675 (1964).



Vitatron, Great Britain) with a voltage control unit (Multirange Module B) for variation of y-scale amplification. We have found it useful to modify the aforementioned equipment on two points. First, for sensitivity reasons, it is desirable to have an automatic zero reset that operates after less than one full volume of the 1-ml burette supplied with the equipment, e.g., after 100  $\mu$ l. This can be done by coupling the -2 V signal obtained from the recorder at maximal y-scale deflection to the burette drive through a relay amplifier (SR 32 Angland), to initiate automatic burette filling. Second, in order directly to monitor the *rate* of FFA release, the first derivative of titrant delivery over time can be registered on the recorder. For this purpose the signal from the burette potentiometer is coupled to the differentiating function present in the electrode potential amplifier (DK 10). The output signal is then connected through a  $10^5$   $\mu$ F condenser to the recorder.

Combined glass electrodes, type EA 120 or EA 147, from Metrohm AG, Herisau, Switzerland are used. Incubations are performed in 30-ml disposable polystyrene vials ( $67 \times 26$  mm). The vials are inserted into a thermostatted ( $37 \pm 0.1^\circ$ ) glass vial (Fig. 1). The lower part of the vial is separated from the upper part by a Plexiglas disk which is sealed with O rings to the glass and incubation vials, respectively (Fig. 1). A stream of water-saturated oxygen is supplied as the gas phase above the disk. The cell suspension is mixed with a Teflon-coated magnetic rod.

## Procedure

### *Stock Solutions*

A: Krebs-Ringer, 590 mM NaCl, 24.8 mM KCl, 12.7 mM  $\text{CaCl}_2$ , 5.95 mM  $\text{KH}_2\text{PO}_4$ , 5.95 mM  $\text{MgSO}_4$ ; diluted 5 times to obtain working solutions

B: Krebs-Ringer modified for pH-stat incubation; as above, but with 655 mM NaCl

C: 2[4-(2-hydroxyethyl)piperazinyl-(1)]ethanesulfonic acid (HEPES), 150 mM; diluted to 24 mM in working solutions

D: Glucose, 140 mM

E: Albumin, 70 g/liter. Bovine serum albumin fraction V is dialyzed at  $4^\circ$  against 20 volumes of redistilled water for  $4 \times 6$  hr, adjusted to pH 7.40 with NaOH, and filtered through a 0.8  $\mu$ m Millipore filter.

Stock solutions are stored at  $-20^\circ$ . The cell media are prepared daily from these solutions.

### *Cell Media*

Krebs-Ringer-HEPES buffer, prepared by dilution of solutions A and C, with the indicated concentrations of albumin, glucose, and collagenase. pH is adjusted to 7.40 with NaOH.

pH-stat incubation medium, prepared by dilution of solution B, with the indicated concentrations of albumin and glucose.

*Preparation and Treatment of Adipocytes.* Intact rat adipocytes are prepared as described previously,<sup>4</sup> except as follows: For each rat, weighing 120–140 g, whole epididymal or perirenal fat (no mincing) is digested in 1.5 ml of Krebs-Ringer-HEPES buffer with 3.5% (w/v) albumin and 0.5 mM glucose containing 0.5 mg of collagenase per milliliter for 90 min, with reciprocal shaking at 155 cycles/min. Released cells are filtered twice through gauze and washed five times in the same medium, but with 1% albumin and no collagenase. Flotation of the cells is facilitated by centrifugation (300 *g*, 20 sec) in polyethylene tubes previously fitted with thin polyethylene tubing. The buffer below the cells is aspirated with a syringe.

The cell concentration is rapidly measured as packed cell volume (PCV) by centrifugation of samples of the cell suspension in microhematocrit tubes and is then diluted to 50  $\mu$ l of PCV per milliliter in the same medium. Two-milliliter samples (taken during thorough mixing to obtain homogeneous cell suspension) can be stored for up to 4 hr in polyethylene counting vials in a water bath with gentle reciprocal shaking (80 cycles/min) without any change in the lipolytic response to norepinephrine.<sup>2</sup>

Since the collagenase preparations differ significantly from batch to batch,<sup>4</sup> cell quality, especially insulin responsiveness, is routinely checked by measuring the ability of the cells to convert medium glucose into intracellular toluene-extractable lipids. Cells (5  $\mu$ l PCV/ml) are incubated for 2 hr in 1 ml of Krebs-Ringer-HEPES buffer with 1% albumin, 0.5 mM glucose, and 0.1  $\mu$ Ci/ml of [ $^3$ H]glucose (0.18 mCi/mmol) with different concentrations of insulin. The incubations are interrupted, and lipids are extracted by adding 10 ml of liquid scintillator: 0.3 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)benzene][(dimethyl-POPOP) and 5 g of 2,5-diphenyloxazole (PPO) per liter of toluene; they are allowed to stand for 1 hr at room temperature, then  $^3$ H-labeled lipids in the upper phase are counted by liquid scintillation.<sup>5</sup> A high concentration of insulin (100  $\mu$ U/ml) should increase glucose incorporation into lipids at least 10-fold.

*pH-Stat Titration of FFA Release.* The cell suspension is first transferred to a polyethylene centrifuge tube as described for washing cells,

<sup>4</sup> J. N. Fain, this series, Vol. 35 [53].

<sup>5</sup> A. J. Moody, M. A. Stan, M. Stan, and J. Gliemann, *Horm. Metab. Res.* 6, 12 (1974).

and the medium is exchanged for pH-stat incubation medium with the desired concentration of glucose and albumin. The low buffer capacity of this incubation medium is important for the sensitivity of the method when using a low cell concentration (10  $\mu$ l of PCV per milliliter in the standard incubation volume of 10 ml), especially at low rates of lipolysis. An occupancy of  $>2$  mol of FFA per mole of albumin inhibits lipolysis,<sup>6</sup> but the albumin also increases the buffer capacity of the medium. Therefore a choice has to be made in each case between maximum sensitivity and ability to measure high lipolysis rates. For most purposes an albumin concentration of 1% (w/v) is useful.

The settings of the pH-stat apparatus and the recorder and the concentration of the titrant have to be established in separate test experiments, conveniently by controlled infusion of  $H^+$  into the incubation vial containing medium as described above. The lipolysis rates obtained by incubation of the described rat fat cells vary from  $<30$  nmol of FFA/ml PCV per minute in the basal (nonstimulated) state to about 2.5  $\mu$ mol of FFA/ml PCV per minute for maximally (100 ng/ml) norepinephrine-stimulated cells. Additions to the incubation vial are made at 37° to avoid temperature-dependent pH changes. The magnetic stirring rate must be constant and should be set at the lowest value that prevents flotation in order not to damage the cells (250 rpm with a  $4 \times 19$  mm magnetic bar is generally sufficient).

After addition of the cells, the pH stat is started and the system is allowed to equilibrate for about 5 mins. During this time titration of FFA, which have, presumably, accumulated intracellularly during the handling of the adipocytes, takes place. After stable conditions have been established, the basal rate of FFA release is registered. Additions (lipolytic hormones, drugs, etc.) to the incubation vial are then made, and the rate of FFA release is registered over an appropriate time period. It is essential that any added component be adjusted to exactly pH 7.40. Several short-time incubations (15–60 min) can usually be made from one cell batch.

Electrode sensitivity is maintained by treating the electrode with pepsin, 5% in 0.1 M HCl, after each experimental day. The sensitivity should be tested regularly by addition of palmitic acid in ethanol to an incubation without cells. The apparatus should give a rapid and constant response to repeated additions of 100 nmol.

### Comments on the Method and Applications

The time course of effects on FFA release from exposure of fat cells to various amounts of norepinephrine are illustrated in Fig. 2 (A, cumulative

<sup>6</sup> J. N. Fain and R. E. Shpherd, *J. Biol. Chem.* **250**, 6586 (1975).

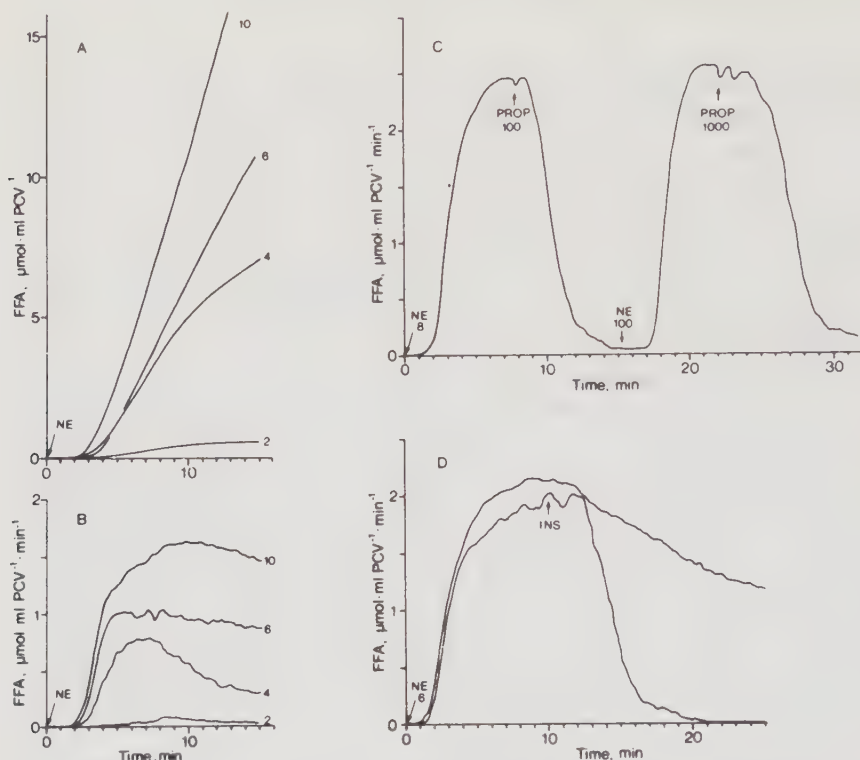


FIG. 2. Release of free fatty acids (FFA) from intact rat adipocytes measured by pH-stat titration. Recorder chart tracings are of FFA release from fat cells, 10  $\mu$ l of packed cell volume (PCV) per milliliter, suspended in 10 ml of pH-stat incubation medium with 1% (w/v) bovine serum albumin and no glucose. NaOH, 5 mM, was used as titrant, and cumulative values (A) or rates (B, C, D) were recorded. Arrows indicate the time point for addition of norepinephrine (NE), propranolol (PROP), and insulin (INS), respectively, as described in what follows. Numbers represent concentrations (ng/ml) for norepinephrine or propranolol. (A, B) Effect of different concentrations of norepinephrine. No measurable FFA release was found in the absence of the hormone. (C) Effect of repeated stimulation of FFA release by norepinephrine addition followed by inhibition of this release by propranolol, a  $\beta$ -adrenergic blocking agent. (D) Effect of insulin (10  $\mu$ U/ml) on norepinephrine-stimulated FFA release compared to a corresponding experiment with no insulin.

values; B, rates). The repeated effects from a  $\beta$ -adrenergic blocking agent, propranolol, on norepinephrine-stimulated FFA release is shown in Fig. 2C and the corresponding effects of insulin in Fig. 2D. The FFA release monitored in these experiments is also a measure of the intracellular lipolysis of stored lipid, since reesterification is insignificant under the conditions used.<sup>2</sup> The FFA release rate therefore directly reflects the ac-

tivity of hormone-sensitive lipase, the enzyme catalyzing the rate-limiting reaction in the lipolysis of adipocyte lipids.<sup>7</sup>

The method is excellently suited for short-time kinetic studies of the effects of hormones, metabolites, and drugs affecting the FFA mobilization from adipocytes. Besides rat cells it has also been used with hamster (unpublished data) and human<sup>2</sup> adipocytes. Samples of the cell suspension can easily be taken during the pH-stat incubation, the cells can be separated from medium within seconds by flotation through silicon oil in small centrifuge tubes<sup>8</sup> and analyzed for intracellular enzymes or metabolites. Using this technique in combination with pH-stat titration, it has been demonstrated that norepinephrine and insulin can regulate lipolysis rate in intact rat adipocytes by altering the extent of phosphorylation of hormone-sensitive lipase.<sup>8</sup>

<sup>7</sup> G. Fredrikson, P. Strålfors, N. Ö. Nilsson, and P. Belfrage, this series, Vol. 71 [74].

<sup>8</sup> N. Ö. Nilsson, P. Strålfors, G. Fredrikson, and P. Belfrage, *FEBS Lett.* **111**, 125 (1980).



## REGULATION OF ADIPOSE TISSUE LIPOLYSIS: PHOSPHORYLATION OF HORMONE-SENSITIVE LIPASE IN INTACT RAT ADIPOCYTES

Per BELFRAGE, Gudrun FREDRIKSON, Nils Östen NILSSON and Peter STRÄLFORS

*Department of Physiological Chemistry 4, University of Lund, POB 750, S-220 07 Lund, Sweden*

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### 1. Introduction

The rate-limiting step in the hydrolysis of adipose tissue triacylglycerols is catalyzed by the hormone-sensitive lipase [1]. It has been suggested that the activity of this enzyme is regulated by its phosphorylation, mediated by hormonal alterations of the intracellular concentration of cyclic AMP [2]. Although likely to be correct, this hypothesis has been based on rather indirect experimental evidence. It is, however, supported by our recent results: the identification of the hormone-sensitive lipase protein and the demonstration that the enzyme can be phosphorylated, and activated, *in vitro* with a cyclic AMP-dependent protein kinase [3,4]. These findings are not necessarily relevant for conditions in the living cell. To assert the physiological relevance of the phosphorylation of the isolated enzyme its phosphorylation must be demonstrated also in the intact adipocyte, i.e., under *in vivo* conditions.

We are studying mechanisms for the hormonal regulation of hormone-sensitive lipase, at the cellular and at the molecular level. Here we have incubated intact rat adipocytes with  $^{32}\text{P}_i$  under conditions giving maximal rate of lipolysis (noradrenaline stimulation). Using a recently developed procedure for extensive purification of hormone-sensitive lipase at a preparative scale [5] we have isolated the enzyme from the adipocytes and shown that it is phosphorylated. In [6] we describe how exposure of the adipocytes to hor-

mones alters the extent of phosphorylation of the enzyme.

### 2. Methods

#### 2.1. Adipocyte preparation, incubation with $^{32}\text{P}_i$ and monitoring FFA release

Intact adipocytes (male Sprague-Dawley rats, 120–140 g) were prepared by collagenase digestion [7,8], amounts of cells given as PCV and their viability evaluated [7,8]. Cells from 25–35 rats were incubated in 25 ml Krebs-Ringer buffer with lower phosphate concentration (50  $\mu\text{M}$ ), containing 20 mM Hepes, 3.5% (w/v) bovine serum albumin and 5 mM glucose at pH 7.40 and 37°C under  $\text{O}_2$  in a pH-stat titration apparatus used for continuous monitoring of FFA release [8]. Carrier-free  $^{32}\text{P}$  orthophosphate (The Radiochemical Centre, Amersham) was added to 125  $\mu\text{Ci/ml}$  final conc. After 40 min incubation noradrenaline (1  $\mu\text{g/ml}$  final conc.) was added for an additional 5 min. FFA release rate increased 25-fold within 2 min of the addition of the hormone, showing the marked stimulation of hormone-sensitive lipase activity (cf. [6,8]).

#### 2.2. Isolation of hormone-sensitive lipase

At the end of the incubation the adipocytes were separated from the bulk of  $^{32}\text{P}_i$ , within 5 min, by floating and resuspending twice in suspension medium containing: 0.25 M sucrose, 1 mM EDTA, 1 mM dithioerythritol, 2 mM ATP, and 0.5  $\mu\text{g/ml}$  noradrenaline at 10°C. NaF was not added, since the partition of the enzyme between the floating fat and 110 000  $\times$  g supernatant (see below) was very sensitive to increased ionic strength [5], and it strongly

**Abbreviations:** cyclic AMP, cyclic 3',5'-adenosinemonophosphate; FFA, free fatty acids; Hepes, 2-[4-(2-hydroxyethyl)-piperazinyl(1)]-ethane sulfonic acid; PCV, packed cell volume; SDS-PAGE, sodium dodecylsulphate-polyacrylamide gel electrophoresis



inhibited enzyme activity [3]. The cells were homogenized in 3 vol. ice-cold suspension medium in a Potter-Elvehjem type glass homogenizer. The fat was floated (1500 rev./min  $\times$  1 min), homogenized again in 1.5 vol. fresh suspension medium and the combined homogenates centrifuged 110 000  $\times$  g for 45 min at 4°C. The supernatant below the fat cake was removed by aspiration and filtered through glass-wool.

The hormone-sensitive lipase was isolated as will be detailed in [5]. In brief: the 110 000  $\times$  g supernatant was brought to pH 5.2 with 0.2 M acetic acid, the precipitate formed collected by centrifugation and suspended in 2.0 ml 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM dithioerythritol (pH 5.2, precipitate fraction). The hormone-sensitive lipase was solubilized in nonionic detergent, C<sub>13</sub>E<sub>12</sub>\* and subjected to gradient sievortive chromatography on QAE-Sephadex (Pharmacia, Uppsala). The pooled enzyme peak fractions were concentrated, and the detergent exchanged for C<sub>8</sub>E<sub>6</sub> by chromatography on spheroidal hydroxylapatite (BDH) followed by adsorption chromatography on Ultrogel AcA 34 (LKB). Details will be given in the figure legends.

Hormone-sensitive lipase purified from epididymal fat pads was [<sup>32</sup>P]phosphorylated with the catalytic subunit of cyclic AMP-dependent protein kinase, purified from the same tissue, by incubation for 30 min at 37°C in 0.1 M Tris-HCl (pH 7.4), 10 mM MgCl<sub>2</sub>, 1 mM dithioerythritol and 0.1 mM [ $\gamma$ -<sup>32</sup>P]-ATP [5].

### 2.3. Analytical procedures

ATP-citrate lyase activity was measured by the malate dehydrogenase-coupled procedure [9]. [<sup>32</sup>P]Protein samples were precipitated and washed with trichloroacetic acid, extracted with diethyl-ether-ethanol [10] and dissolved with SDS under reducing conditions by sonication and heating [6]. Samples from adipocyte suspensions were first delipidated [6]. SDS-PAGE was according to [11] with modifications [6] in 14 cm slabgels with 8% acrylamide using phosphorylase *a* from rabbit muscle (94 000 daltons), human transferrin (76 700 daltons), bovine serum albumin (67 000 daltons), catalase from bovine liver (60 000 daltons), ovalbumin

(43 000 daltons) and lactate dehydrogenase from rabbit muscle (34 000 daltons) as molecular weight markers. After kenacid blue (BDH) staining, gels were dried and autoradiographed on Osray M 3 films (Agfa-Gevaert). [<sup>32</sup>P]Protein bands were quantitated by scanning densitometry at 633 nm (soft laser densitometer, model SL 504, Biomed Instr., Chicago, IL) of autoradiographs and calculation of relative peak areas. Protein was determined by a scaled-down version [12] of the Lowry method [13].

## 3. Results

### 3.1. Experimental outline

The experiment was designed to isolate hormone-sensitive lipase from protein extracts of adipocytes, incubated with <sup>32</sup>P<sub>i</sub>, by a procedure developed for preparative-scale purification of the enzyme from adipose tissue [5], which results in an enzyme of >50% protein purity by SDS-PAGE (app. mol. wt 84 000) [5]. The presence of <sup>32</sup>P<sub>i</sub> in enzyme, thus isolated from adipocytes, has been investigated for evidence for the phosphorylation of hormone-sensitive lipase in intact cells. Six similar experiments have been performed with the same general results.

### 3.2. Initial fractionation of adipocyte proteins, precipitation of the lipase at pH 5.2 and gradient sievortive chromatography on QAE-Sephadex

The fat-depleted 110 000  $\times$  g supernatant contained part of the hormone-sensitive lipase from the adipocyte homogenate. Practically no enzyme was sedimented but part of it remained bound to the floated fat as indicated by pH-stat titration (presence of endogenous lipids made enzyme determination with radiolabeled substrate [14] impossible). The lipase activity of the 110 000  $\times$  g supernatant was almost quantitatively recovered in the pH 5.2 precipitate fraction with 1.5-fold increase of specific activity. Adipocyte protein extracts contained a large number of [<sup>32</sup>P]phosphopeptides resolved by SDS-PAGE (fig.1, insert a). Most of these [<sup>32</sup>P]phosphopeptides were also found in the pH 5.2 precipitate fraction (fig.1, insert b). A small 84 000 dalton [<sup>32</sup>P]phosphopeptide band was present in both fractions. It was also found in samples from the isolated floating fat layer, but not from the 110 000  $\times$  g pellet and pH 5.2 supernatant (not illustrated).

Further fractionation of the hormone-sensitive

\* The nonionic detergents used were polydisperse preparations of alkyl polyoxyethylene glycol. They are designated C<sub>x</sub>E<sub>y</sub>, where *x* = average number of alkyl chain carbons and *y* = oxyethylene units

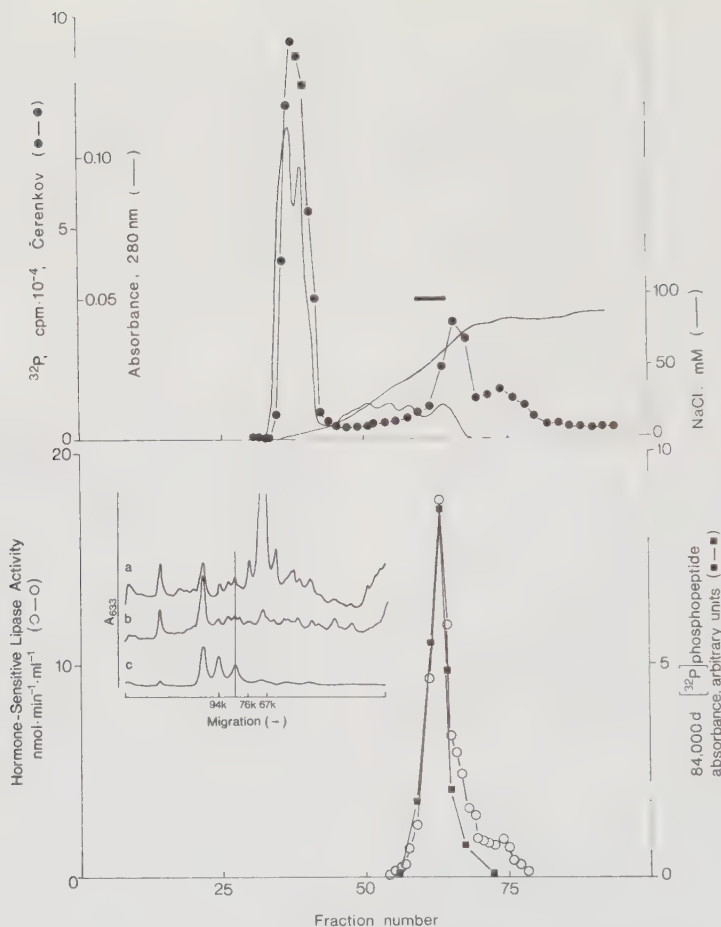
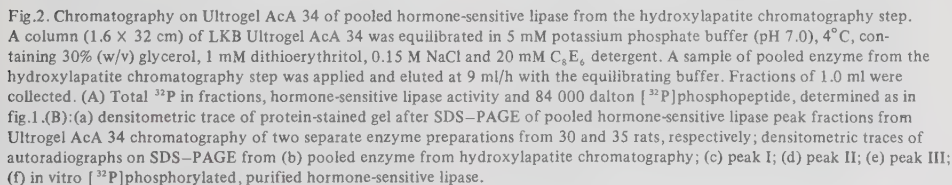


Fig.1. Gradient sievortype chromatography on QAE-Sephadex of detergent-solubilized (pH 5.2) precipitate fraction. A QAE-Sephadex A-25 column (2.6  $\times$  91 cm) was equilibrated at pH 8.05, 10°C in 40 mM Tris-HCl containing 20% (w/v) glycerol, 0.2 mM EDTA, 1 mM dithioerythritol and 0.2%  $\text{C}_{13}\text{E}_{12}$  detergent. A 32 ml 0–85 mM NaCl linear gradient was applied immediately before the sample: 4.0 ml detergent-solubilized (pH 5.2) precipitate fraction obtained from 6.2 ml PCV of fat cells from 25 rats, containing 85 mM NaCl, 13% sucrose, 1 mM EDTA, 1 mM dithioerythritol and 6.3%  $\text{C}_{13}\text{E}_{12}$ . The column was eluted with 85 mM NaCl in the equilibrating buffer at 10°C at a flow-rate of 5.0 ml/h  $\cdot \text{cm}^2$ . Fractions of (4.3 ml) were collected. Hormone-sensitive lipase activity was measured at 37°C with emulsified 1(3)-oleoyl-2-oleylglycerol as substrate [14].  $[\text{NaCl}]$  and  $A_{280}$  were continuously monitored. The bar indicates the fractions pooled.  $^{32}\text{P}$  radioactivity was measured as total Čerenkov radiation from SDS-PAGE and calculation of the peak areas.

Insert: densitometric traces of autoradiographs of SDS-PAGE of: (a) adipocyte suspension; (b) pH 5.2 precipitate fraction; (c) pooled enzyme fractions from QAE-Sephadex chromatography. Molecular weight of marker proteins as indicated,  $k = 10^3$ . Migration position of 84 000 dalton  $^{32}\text{P}$  phosphopeptide indicated by vertical line.

At the later step the enzyme adsorbed and was eluted with high phosphate concentration (present in the applied sample) (fig.2A). Since  $<0.1$  mg protein was applied the  $A_{280}$  elution pattern could not be followed. However, SDS-PAGE on consecutive fractions indicated that practically all contaminating protein was eluted before hormone-sensitive lipase, with



some minor contaminants in the ascending slope of enzyme activity. Densitometric trace of protein stain from SDS-PAGE of enzyme peak fractions pooled from 2 preceding experiments indicated a protein purity of ~35% for the hormone-sensitive lipase 84 000 dalton polypeptide (fig.2B,a). Overall recovery of enzyme activity compared to the 110 000 X g supernatant was 6%. A peak of  $^{32}\text{P}$ -radioactivity (III) was eluted with the enzyme activity, well resolved from 2 other peaks, which were due to [ $^{32}\text{P}$ ]ATP-citrate lyase (I) and, mainly, the 94 000 dalton [ $^{32}\text{P}$ ]phosphopeptide (II) (fig.2). Peak III  $^{32}\text{P}$  radioactivity was almost entirely due to the 84 000 dalton [ $^{32}\text{P}$ ]phosphopeptide (fig.2B,e). This [ $^{32}\text{P}$ ]phosphopeptide had the same mobility (SDS-PAGE) as purified, *in vitro* [ $^{32}\text{P}$ ]phosphorylated, hormone-sensitive lipase (fig.2B,f). The identity was verified by the approximately constant relation between enzyme activity and  $^{32}\text{P}$  radioactivity in the 84 000 dalton polypeptide over the enzyme activity peak (fig.2A).

#### 4. Discussion

These results show that hormone-sensitive lipase is phosphorylated in intact rat adipocytes. The copurification of the 84 000 dalton [ $^{32}\text{P}$ ]phosphopeptide with enzyme activity over several isolation steps, to an enzyme protein purity of 35%, is strong evidence for its identification with hormone-sensitive lipase. The [ $^{32}\text{P}$ ]phosphorylation of the enzyme occurred within the fat cells and not during the isolation procedure since, when the cells were broken, the ATP in the homogenisation medium immediately diluted the [ $\gamma$ , $^{32}\text{P}$ ]ATP in the adipocytes. Moreover, [ $^{32}\text{P}$ ]phosphorylated enzyme was obtained when cells were extracted with SDS solution, within seconds of separation from the incubation medium [6].

[ $^{32}\text{P}$ ]Hormone-sensitive lipase seemed to be the main constituent of the 84 000 dalton [ $^{32}\text{P}$ ]phosphopeptide band obtained by SDS-PAGE of adipocyte proteins. This [ $^{32}\text{P}$ ]phosphopeptide band was only obtained from fractions containing enzyme; the only significant amount not used in the isolation of the enzyme was found in the floating fat layer.

The demonstration that hormone-sensitive lipase can be phosphorylated in the intact cell is required for, but does not prove, a physiological role for this

process in the hormonal regulation of enzyme activity. The effects of hormones on the phosphorylation will be described in [6].

#### Acknowledgements

Mrs Birgitta Danielsson and Mrs Ingrid Nordh gave skillful technical assistance. The project was supported by grants from Pålssons Foundation, Malmö, Segerfalks Foundation, Helsingborg, Novo Insulin Foundation, Copenhagen, the Swedish Diabetes Foundation, Swärds Foundation, Stockholm, The Medical Faculty of the University of Lund and the Swedish Medical Research Council (grant no. 3362).

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## REGULATION OF ADIPOSE TISSUE LIPOLYSIS: EFFECTS OF NORADRENALINE AND INSULIN ON PHOSPHORYLATION OF HORMONE-SENSITIVE LIPASE AND ON LIPOLYSIS IN INTACT RAT ADIPOCYTES

Nils Östen NILSSON, Peter STRÅLFORS, Gudrun FREDRIKSON and Per BELFRAGE

*Department of Physiological Chemistry 4, University of Lund, POB 750, S-220 07 Lund, Sweden*

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### 1. Introduction

In [1] we have demonstrated that hormone-sensitive lipase is phosphorylated in intact rat adipocytes, but the effects of hormones on the extent of this phosphorylation was not studied. The [ $^{32}\text{P}$ ]phosphorylated enzyme protein migrated in a small [ $^{32}\text{P}$ ]phosphopeptide band (1.5% of total) when proteins from adipocytes, incubated with  $^{32}\text{P}_i$ , were separated with SDS-PAGE. During the isolation of the [ $^{32}\text{P}$ ]phosphorylated lipase we obtained no indication of heterogeneity of this 84 000 dalton [ $^{32}\text{P}$ ]phosphopeptide band, which suggested that it mainly consisted of [ $^{32}\text{P}$ ]hormone-sensitive lipase [1].

Based on these observations we have derived a method for quantitation of [ $^{32}\text{P}$ ]hormone-sensitive lipase in adipocyte protein extracts. We had developed a technique for continuous monitoring of FFA release from adipocytes by pH-stat titration as a measure of hormone-sensitive lipase activity [2]. In combination, these techniques have enabled us to correlate the time-course of effects of noradrenaline and insulin on phosphorylation and activity of the enzyme, in intact adipocytes. The results suggest that these hormones can regulate lipolysis in the intact cell by altering the extent of phosphorylation of hormone-sensitive lipase.

### 2. Methods

#### 2.1. Quantitation of [ $^{32}\text{P}$ ]hormone-sensitive lipase in adipocyte protein extracts

Rat adipocytes [1] were incubated under conditions given in the figure legends. Adipocytes were separated from incubation medium by floatation (10 000  $\times g$  for 5 s, Beckman Microfuge B, 0.4 ml tubes) through dinonylphthalate (BDH) [3]. In <15 s the floated cells were dissolved, in the tubes, in 50  $\mu\text{l}$  solution containing 3% SDS, 0.1 M 2-mercaptoethanol, 50 mM  $\text{NaP}_i$ , 10 mM EDTA, 10 mM NaF, and 10% (v/v) ethanol (pH 8.5). The tubes were stored at  $-20^\circ\text{C}$ . For analysis by SDS-PAGE tubes were sliced and cell proteins (100–150  $\mu\text{g}$ ) precipitated in ice-cold acetone, extracted with organic solvents [4], and dissolved in 100  $\mu\text{l}$  electrophoresis sample solution (1.25% SDS, 1 mM EDTA, 63 mM Tris-HCl (pH 8.6), 10% (w/v) glycerol, 5% (w/v) 2-mercaptoethanol and 20 mg/l bromphenol blue) by repeated heating to  $96^\circ\text{C}$  for 1.5 min, sonication for 10 min and vortex mixing for 30 s.

SDS-PAGE was done following [5] but with 2 mM EDTA in all solutions [6], electrode buffer (pH 8.7) and acrylamide at  $8 \times 2.4\%$  (T  $\times$  C). Kenacid blue (BDH) stained gels were dried and autoradiographed using Osray M3 films (Agfa-Gevaert). Gels and autoradiographs were scanned with a soft laser densitometer at 633 nm (model SL 504, Biomed Instr., Chicago, IL). Radioactivity in the hormone-sensitive lipase band was quantitated by liquid scintillation counting. After localization of the band, with the autoradiograph, a gel piece (2.5  $\times$  12 mm) was punched out from the dried gel, dissolved in

**Abbreviations:** ATP, adenosine 5'-triphosphate; cyclic AMP, cyclic 3',5'-adenosinemonophosphate; Hepes, 2-[4-(2-hydroxyethyl)-piperazinyl-(1)]-ethane sulfonic acid; FFA, free fatty acid; PCV, packed cell volume; SDS-PAGE, sodium dodecylsulphate-polyacrylamide gel electrophoresis



1 ml 30%  $\text{H}_2\text{O}_2$ , and Instagel (Packard) added. Background radioactivity was measured, in gel pieces of equal size from an area where no phosphopeptide was visible, and subtracted. Variation in amount of protein applied to the gels was <10% and not corrected for.

## 2.2. Hydroxylapatite chromatography of SDS-protein complexes

Autoradiographs (Kodak X-Omat R film, Du Pont Cronex intensifying screen, 3 h exposure) of wet, unstained SDS-PAGE gels were used to localize the 84 000 dalton [ $^{32}\text{P}$ ]phosphopeptide band. This band was cut out from the gel and the [ $^{32}\text{P}$ ]phosphopeptides eluted electrophoretically into a dialysis bag as in [7]. The eluates (1–2 ml) were dialysed for 2 h against 10 mM  $\text{NaP}_i$ , 0.1% SDS, and 1 mM dithio-

erythritol (pH 6.4) and fractionated on hydroxylapatite (HTP, Bio-Rad) according to [8].

## 2.3. Analytical procedures and materials

Intracellular ATP was determined on perchloric acid extracts of floated cells by an enzymatic fluorometric method [9]. [ $^{32}\text{P}$ ]ATP in the same extracts was determined by liquid scintillation counting after thin-layer chromatography on poly(ethyleneimine)-cellulose (Merck) [10]. L-Noradrenaline bitartrate and bovine serum albumin, fraction V (extensively dialysed before use) were from Sigma Chemical Co., insulin (porcine, monocomponent, 26.3 units/mg) was a gift from Novo, acrylamide from Bio-Rad and carrier-free [ $^{32}\text{P}$ ]orthophosphate from The Radiochemical Centre (Amersham).



Fig.1. Adipocyte proteins and [ $^{32}\text{P}$ ]phosphopeptides fractionated by SDS-PAGE. Adipocytes (50  $\mu\text{l}$  PCV/ml) were incubated in Krebs-Ringer buffer with low phosphate (50  $\mu\text{M}$ ) containing 3.5% (w/v) bovine serum albumin, 5 mM glucose and 100  $\mu\text{Ci/ml}$  carrier-free  $^{32}\text{P}_i$  for 40 min and, when indicated, with noradrenaline or noradrenaline followed by insulin. Delipidated cell protein (100–150  $\mu\text{g}$ ) was separated with SDS-PAGE on slab gels. (a) Protein stain of adipocyte protein extract. Autoradiograph and densitometric trace of; (b,c) purified, *in vitro* [ $^{32}\text{P}$ ]phosphorylated hormone-sensitive lipase [11]; (c,f) [ $^{32}\text{P}$ ]phosphopeptides from adipocytes exposed to noradrenaline (50 ng/ml =  $2.95 \times 10^{-7}$  M for 5 min); (d,g) [ $^{32}\text{P}$ ]hormone-sensitive lipase isolated from cells incubated with  $^{32}\text{P}_i$  [1]; (h) 84 000 dalton region of densitometric traces as in (c,f) where control = incubation without hormones, NA = addition of noradrenaline (50 ng/ml) for 5 min, and NA + INS = addition of insulin (100  $\mu\text{units/ml}$ ) for 10 min after the 5 min incubation with noradrenaline. Migrating positions of molecular weight marker proteins [1] and 84 000 dalton [ $^{32}\text{P}$ ]phosphopeptide band have been indicated in the figure ( $k = 10^3$ ).

### 3. Results

#### 3.1. Quantitation of [ $^{32}\text{P}$ ]hormone-sensitive lipase in adipocyte protein extracts

Protein from intact adipocytes, incubated with  $^{32}\text{P}_i$  and noradrenaline, was separated with SDS-PAGE and the gels autoradiographed. The 84 000 dalton [ $^{32}\text{P}$ ]phosphopeptide band, containing [ $^{32}\text{P}$ ]hormone-sensitive lipase (identified in fig.1c,f), had the same electrophoretic mobility as a very small protein band (order of magnitude: 0.1% of total) on the protein-stained gel (fig.1a). Hormone effects on the height of the 84 000 dalton [ $^{32}\text{P}$ ]phosphopeptide peak were observed (fig.1h).

To demonstrate that the hormone effects were due to changes in the extent of phosphorylation of hormone-sensitive lipase the [ $^{32}\text{P}$ ]phosphopeptide-SDS complexes of the 84 000 dalton band were further fractionated by hydroxylapatite chromatography. As reference proteins [ $^{32}\text{P}$ ]hormone-sensitive lipase, after purification from rat adipose tissue and phosphorylation *in vitro* [11], or after isolation from intact adipocytes incubated with  $^{32}\text{P}_i$  [1], were used. The former (fig.2A) and the latter (not illustrated) reference [ $^{32}\text{P}$ ]proteins were eluted at 0.48 M phosphate, coinciding with the major [ $^{32}\text{P}$ ]phosphopeptide peak from the 84 000 dalton band (fig.2B-D). This peak was thus identified as [ $^{32}\text{P}$ ]hormone-sensitive lipase. Changes of  $^{32}\text{P}$  in the 84 000 dalton band, due to exposure of the adipocytes to hormones, were found almost entirely in this [ $^{32}\text{P}$ ]hormone-sensitive lipase peak. These results verify that most of the 84 000 dalton [ $^{32}\text{P}$ ]phosphopeptide SDS-PAGE band from adipocyte protein extracts consists of [ $^{32}\text{P}$ ]hormone-sensitive lipase, that the [ $^{32}\text{P}$ ]enzyme can be approximately

quantitated by determination of the  $^{32}\text{P}$  of this band and, thus, that hormone alterations of  $^{32}\text{P}$  in this band reflect changes in the extent of [ $^{32}\text{P}$ ]phosphorylation of hormone-sensitive lipase.

[ $^{32}\text{P}$ ]Hormone-sensitive lipase could be quantitated either by determination of densitometric peak height,

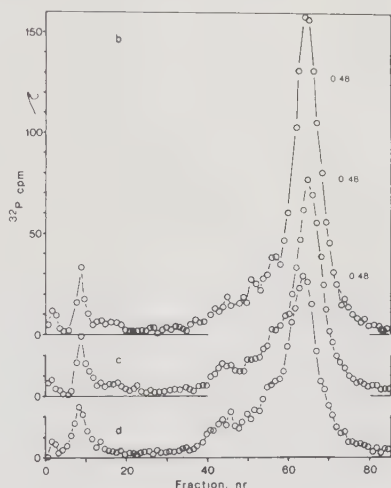
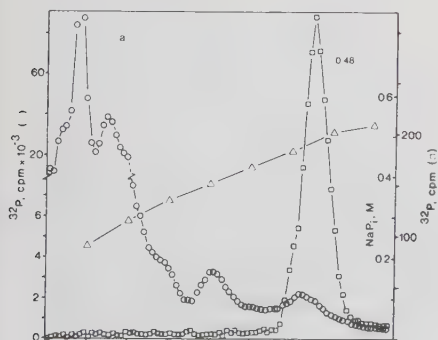


Fig.2. Hydroxylapatite chromatography of [ $^{32}\text{P}$ ]phosphopeptide-SDS complexes. [ $^{32}\text{P}$ ]Phosphopeptides, eluted electrophoretically from SDS-PAGE gel slices or in an adipocyte protein extract, were dialyzed against 10 mM sodium phosphate buffer (pH 6.5) containing 0.1% SDS, and 1 mM dithioerythritol. They were then applied to hydroxylapatite columns ( $10 \times 0.7$  cm), pre-equilibrated in the dialyzing buffer, and eluted at  $27 \pm 1^\circ\text{C}$  with a 0.20–0.50 M sodium phosphate gradient (pH 6.5) with SDS and dithioerythritol as above. Flowrate was 7.0 ml/h and fraction vol. 0.7 ml. Delipidated adipocyte protein (100  $\mu\text{g}$ ) was added to obtain an equal amount of protein in each sample applied. Sodium phosphate concentration was determined by conductivity, and  $^{32}\text{P}$  radioactivity as Cerenkov-radiation. (a) ( $\circ$ — $\circ$ ) delipidated adipocyte protein (100  $\mu\text{g}$ ) extract; ( $\square$ — $\square$ ) [ $^{32}\text{P}$ ]hormone-sensitive lipase after SDS-PAGE, the enzyme had been phosphorylated *in vitro* after purification from adipose tissue [11]; (b) 84 000 dalton [ $^{32}\text{P}$ ]phosphopeptide band from protein extract of adipocytes, incubated as in fig.1, noradrenaline 50 ng/ml for 5 min; (c) as in (b) but cells incubated with noradrenaline (50 ng/ml, 5 min) followed by insulin, 100  $\mu\text{units/ml}$  for 10 min; (d) as in (b) but cells incubated without hormones. Phosphate gradient is plotted for adipocyte protein extract only. Values in the figure denote phosphate molarity in peak fractions. Fraction numbers are not exactly related to phosphate concentration.



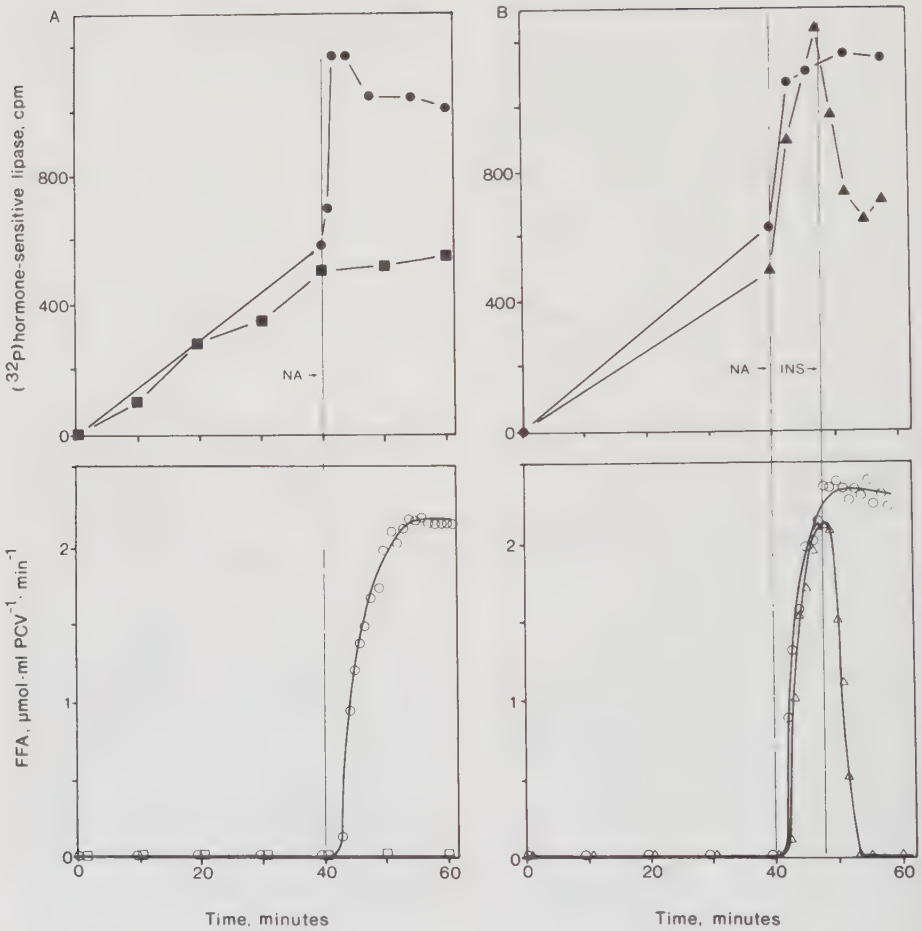


Fig.3. Effect of noradrenaline and insulin on the time-course of  $[^{32}\text{P}]$ phosphorylation of hormone-sensitive lipase and on lipolysis rate. Adipocytes were incubated as in fig.1. Lipolysis, measured as FFA release, was monitored continuously by pH-stat titration [2] and lipolysis rate derived over 1 min intervals. Duplicate samples (0.5 ml) of the adipocyte suspension were taken at different times and the  $[^{32}\text{P}]$ phosphopeptides separated by SDS-PAGE.  $^{32}\text{P}$  radioactivity in hormone-sensitive lipase was determined by liquid scintillation counting; each point is the mean of 2 values. (A) Effect of noradrenaline, 50 ng/ml (NA;  $\bullet$ ,  $\circ$ ) compared to control without hormone ( $\blacksquare$ ,  $\square$ ). (B) Effect of insulin, 100  $\mu\text{units/ml}$  (INS;  $\blacktriangle$ ,  $\triangle$ ) on noradrenaline-stimulated (50 ng/ml) adipocytes compared to noradrenaline-stimulated control ( $\bullet$ ,  $\circ$ ). Closed symbols refer to  $[^{32}\text{P}]$ phosphorylation of hormone-sensitive lipase, open symbols to FFA release rate.

or by direct  $^{32}\text{P}$  counting as in section 2. The latter method was used since it gave higher reproducibility.

### 3.2. Time-course of effect of noradrenaline and insulin on [ $^{32}\text{P}$ ]phosphorylation of hormone-sensitive lipase and on lipolysis rate

Intracellular concentration of ATP was  $123 \pm 4$  (6) nmol/ml PCV (mean  $\pm$  SD, no. cell batches) and varied by  $<9\%$  within each experiment. There was no significant change with time or by addition of hormones during the experiment.  $^{32}\text{P}_i$  was incorporated into cellular [ $^{32}\text{P}$ ]ATP, at a constant rate. After 40 min incubation [ $^{32}\text{P}$ ]ATP had reached an approximately constant specific activity ( $<7\%$  increase over 40–60 min incubation) with no significant effect of addition of noradrenaline or insulin.

In adipocytes incubated without added hormone,  $^{32}\text{P}$  incorporation into hormone-sensitive lipase increased, concomitantly with [ $^{32}\text{P}$ ]ATP specific activity, to a steady state level, with no change in lipolysis rate (fig.3A). Noradrenaline addition, in  $<2$  min increased [ $^{32}\text{P}$ ]phosphorylation and concomitantly, with a slight delay, the activity of the enzyme (FFA release) (fig.3A). In 11 expt the [ $^{32}\text{P}$ ]phosphorylation increased to an average of  $178 \pm 7.2\%$  (SEM)  $p < 0.001$ , of controls. Addition of insulin to maximally noradrenaline-stimulated adipocytes, in  $<4$  min reduced the extent of [ $^{32}\text{P}$ ]phosphorylation of the enzyme, approaching that obtained before noradrenaline-stimulation (fig.3B). This decrease in  $^{32}\text{P}$  content was followed, with a slight delay, by a reduced rate of lipolysis. (This effect on FFA release was due to inhibition of lipolysis and not to an increased re-esterification, since it was not significantly affected by absence of glucose in the incubation.) In 6 similar experiments insulin addition reduced the  $^{32}\text{P}$  content of the enzyme from  $186 \pm 8.1\%$  (SEM) to a level of  $118 \pm 3.3\%$  (SEM) of that obtained before noradrenaline addition. This reduction was statistically significant,  $p < 0.01$  (Student's  $t$ -test for paired observations). The same extent of [ $^{32}\text{P}$ ]phosphorylation, which was obtained after insulin, was not associated with any measurable lipolysis in the dose-response experiments (fig.4, below).

### 3.3. Effects of noradrenaline and insulin concentration on [ $^{32}\text{P}$ ]phosphorylation of hormone-sensitive lipase and lipolysis rate

Increase in extent of [ $^{32}\text{P}$ ]phosphorylation of the

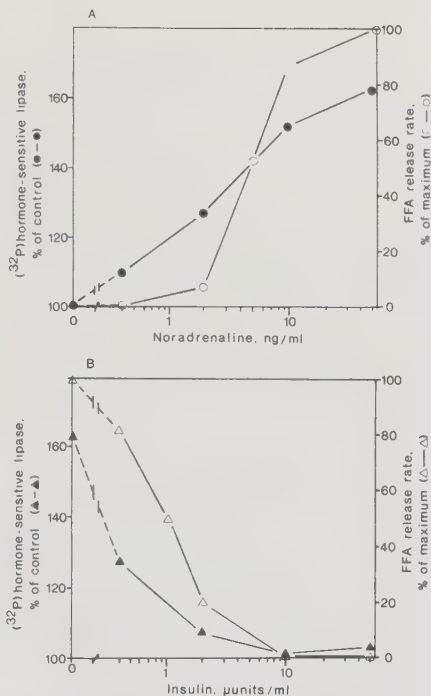


Fig.4. Effects of different concentrations of noradrenaline and insulin on lipolysis rate and [ $^{32}\text{P}$ ]phosphorylation of hormone-sensitive lipase. Adipocytes were incubated as in fig.1, but with Hepes, 24 mM, in 1 ml final vol. (A) Exposure of the cells to the indicated concentrations of noradrenaline for 10 min, after 40 min preincubation with  $^{32}\text{P}_i$ . (B) Exposure of the cells to 10 ng noradrenaline/ml for 10 min as in (A) followed by insulin at the indicated concentrations for 10 min. Closed symbols refer to  $^{32}\text{P}$ -phosphorylation of enzyme, expressed as % of controls without hormone (40 min values); open symbols to FFA release rate. Each value is the mean of 4.

enzyme and rate of lipolysis occurred over the same range of noradrenaline concentrations, with half-maximal effect at 3–5 ng/ml (fig.4A). Decrease in extent of [ $^{32}\text{P}$ ]phosphorylation of the enzyme and rate of lipolysis, pre-stimulated with 10 ng noradrenaline/ml, occurred over the same range of insulin concentrations, with half-maximal effect at 0.5–1  $\mu$ unit/ml (fig.4B).

#### 4. Discussion

These results suggest that the activity of hormone-sensitive lipase in intact adipocytes is regulated by hormonal alterations of the extent of phosphorylation of the enzyme protein. Most of the  $^{32}\text{P}$  radioactivity present in the 84 000 dalton band in the SDS-PAGE gels was due to hormone-sensitive lipase, even though this band also contained some other  $^{32}\text{P}$  phosphopeptides. Moreover, hormones affected mainly, if not exclusively, the  $^{32}\text{P}$  phosphorylation of hormone-sensitive lipase. The enzyme was extensively  $^{32}\text{P}$ -phosphorylated without any effect on lipolysis rate, when the fat cells were incubated in the absence of hormone (fig.3A). This may reflect phosphate exchange without net increase in phosphorylation, but it cannot be excluded that the phosphorylation occurred at a different site not directly affecting enzyme activity (cf. [13]).

Since noradrenaline effects are presumed to be mediated by cyclic AMP-dependent phosphorylation, the present work indicates that the *in vitro* phosphorylation of the purified enzyme with a cyclic AMP-dependent protein kinase [11,12] is physiologically important. An important finding was that insulin inhibition of noradrenaline-stimulated lipolysis was associated with a decrease in the extent of phosphorylation of the enzyme. This suggests one possible mechanism for the action of insulin. It could, however, not be distinguished whether the effects of insulin were mediated by changes in protein kinase activity or phosphoprotein phosphatase activity. The sensitivity of lipolysis and enzyme phosphorylation to insulin concentration was in the same range as that found recently for both lipolysis and glucose uptake in rat adipocytes under similar conditions [14].

The presence of, and hormone effects on, a small  $^{32}\text{P}$  phosphopeptide band, given the app. mol. wt 86 000 dalton, had been noticed, but its identity was not discussed [15]. The reason for not observing the hormone-sensitive lipase  $^{32}\text{P}$  phosphopeptide band in the SDS-PAGE fractionations [16] may be that, at the ionic strength used, the enzyme was not separated from the fat, after homogenization of the adipocytes (cf. [11]).

The continuous monitoring of enzyme activity in intact cells, in combination with determinations of enzyme phosphorylation, makes this system particularly useful for studies of hormonal regulatory mechanisms. The effects of hormones on enzyme

activity and phosphorylation discussed here, certainly suggest a number of important aspects of their mechanism of action that are now being investigated further.

#### Acknowledgements

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THE ANTI-LIPOLYTIC EFFECT OF INSULIN:

INHIBITION OF LIPOLYSIS IN RAT ADIPOCYTES BY  
DECREASED EXTENT OF PHOSPHORYLATION OF HORMONE-SENSITIVE LIPASE

By

Nils Östen Nilsson and Per Belfrage

Department of Physiological Chemistry 4, University of Lund,  
Lund, Sweden



## SUMMARY

The mechanism for the antilipolytic effect of insulin has been studied in intact rat adipocytes using recently developed techniques for determination of the phosphorylation state of hormone-sensitive lipase (HSL) and of the lipolysis rate by continuous pH-stat titration of free fatty acid release. Insulin inhibited noradrenaline-stimulated lipolysis in a concentration-dependent manner. This insulin effect was apparently potentiated by endogenously produced adenosine accumulating during the incubations. Insulin had no effect on the basal phosphorylation of HSL, which indicated that most of this phosphorylation occurred at a site which was not hormonally controlled. When added shortly before, or together with noradrenaline insulin inhibited lipolysis and prevented the noradrenaline-induced increase of the HSL phosphorylation state. When added after noradrenaline insulin decreased lipolysis and the HSL phosphorylation state. The antilipolytic effect of insulin was closely related temporally and quantitatively to the decrease of the HSL phosphorylation state. These findings establish that insulin inhibited lipolysis in intact rat adipocytes through net dephosphorylation, and thereby inactivation, of HSL, confirming and extending our previous results (Nilsson, N.Ö., Strålfors, P., Fredrikson, G. and Belfrage, P. 1980, *FEBS Lett.* 111, 125-130).

The antilipolytic effect was almost four-fold more sensitive to insulin than glucose conversion to adipocyte lipids - which mainly reflects glucose uptake - when both parameters were measured under identical conditions.

Millimolar concentrations of dibutyryl cyclic AMP increased the phosphorylation of HSL and lipolysis rate to the same extent as maximal noradrenaline stimulation, indicating that the effects of this hormone were mediated through increased cellular cyclic AMP. Insulin did not inhibit this maximally dibutyryl cyclic AMP-stimulated lipolysis and HSL phosphorylation. This suggests that a large part of the antilipolytic effect of insulin may be mediated by a cyclic AMP-dependent mechanism.

Abbreviations used are: HSL, hormone-sensitive lipase; FFA, free fatty acids; PCV, packed cell volume; HEPES, 2-[4-(2-hydroxyethyl)-piperazinyl-(1)]-ethane sulfonic acid; SDS-PAGE, sodium dodecylsulphate-polyacrylamide gel electrophoresis; NA, noradrenaline; INS, insulin; Db-cAMP, N<sup>6</sup>-O<sup>2</sup>-dibutyryl cyclic adenosine 3':5'-monophosphate; ATP, adenosine-5'-triphosphate; Ado, adenosine.

## INTRODUCTION

One of the principal biological functions of insulin is to regulate fatty acid mobilization from adipose tissue by inhibiting the rate of lipolysis. This antilipolytic effect of insulin was detected more than 15 years ago with rat adipose tissue preparation (1,2) and can be easily demonstrated with isolated rat adipocytes (3). In spite of this the mechanism of the insulin action has yet to be elucidated.

We have recently purified hormone-sensitive lipase (HSL), the enzyme controlling the rate of lipolysis, from rat adipose tissue (4) and demonstrated that the activity of the isolated enzyme is regulated by phosphorylation, catalyzed by cyclic AMP-dependent protein kinase from the same tissue (4). HSL has also been shown to become phosphorylated in the intact fat cell, after hormonal stimulation (5). A method has been developed for determination of the extent of phosphorylation of the enzyme in intact adipocytes by measuring its  $^{32}\text{P}$ -phosphorylation after preincubation of the fat cells with [ $^{32}\text{P}$ ]orthophosphate (6). In combination with a previously developed method for the direct and continuous monitoring of lipolysis, as FFA release, by pH-stat titration (7), this technique provided evidence indicating that noradrenaline stimulated lipolysis in intact fat cells by phosphorylation of hormone-sensitive lipase (6). The antilipolytic action of insulin seemed to be associated with decreased extent of this phosphorylation (6).

In the present work we have extended these studies on the antilipolytic effect of insulin, employing the same techniques. The results establish that insulin inhibits lipolysis by preventing noradrenaline-stimulated phosphorylation of HSL or by decreasing the extent of HSL phosphorylation induced by previous noradrenaline stimulation.



## METHODS

Preparation of rat adipocytes and incubations

Intact rat adipocytes were prepared from 120-150 g *ad libitum* fed male Sprague-Dawley rats by collagenase (0.5 mg/ml) digestion (7). The packed cell volume (PCV) of the preparation was rapidly determined (8) and the cell suspension diluted to 50  $\mu$ l PCV/ml and stored in a modified Krebs-Ringer medium (7) containing 24 mM HEPES, 1% bovine serum albumin and 0.55 mM glucose at 37°C, with gentle shaking. Immediately before each experiment cells were washed and suspended in fresh incubation medium. The standard incubation medium for  $^{32}$ P-phosphorylation experiments with 50  $\mu$ l PCV of cells per ml in the pH-stat titrator vessels had the following composition: 131 mM NaCl, 4.95 mM KCl, 2.54 mM CaCl<sub>2</sub>, 0.05 mM KH<sub>2</sub>PO<sub>4</sub>, 1.19 mM MgSO<sub>4</sub>, 35 mg/ml bovine serum albumin, pH 7.40. Carrier-free [ $^{32}$ P]-orthophosphate was added to 50  $\mu$ Ci/ml. When incubations were not performed in the pH-stat titrator vessels, 24 mM HEPES was added to control the pH. When so indicated lipolysis experiments were performed with 10  $\mu$ l PCV of cells per ml in the medium described above but with 10 mg/ml bovine serum albumin.

The cell preparation never contained more than 5% (v/v) broken cells. The cell quality was regularly tested as the ability of insulin to stimulate the conversion of medium [ $3\text{-}^3\text{H}$ ]glucose (0.55 mM) to cellular lipids (9). Only cells in which insulin (700 pM) caused at least a 10-fold increase of this parameter, with half-maximal effect at 50-70 pM (5  $\mu$ l PCV/ml incubated at 37°C for 1 h) were used (7). One  $\mu$ l of PCV was equivalent to about  $1.6 \cdot 10^4$  cells from adipose tissue of 120-150 g rats (cell diameter approx. 50  $\mu$ m).

All experiments have been carried out several times with essentially the same results. Due to differences between cell batches, the quantitative responses of the parameters studied to hormones varied somewhat, however, and therefore, rather than attempt to combine data from several different cell prepa-

rations, data from individual representative experiments are reported.

#### Determination of the extent of phosphorylation of hormone-sensitive lipase

The extent of HSL phosphorylation in intact adipocytes was estimated from the  $^{32}\text{P}$ -phosphorylation of the enzyme as in (6). At the appropriate times 0.5 ml samples of the cell suspension were taken for [ $^{32}\text{P}$ ]HSL analysis. Albumin-containing medium was rapidly separated from the cells by 5 sec centrifugation (10,000 *g*) with 100  $\mu\text{l}$  dinonylphthalate in two 400  $\mu\text{l}$  polyethylene tubes. Floated cells were then immediately disrupted in SDS medium (6), delipidated by consecutive extractions with 1.5 ml acetone, 1 ml ethanol, 1 ml chloroform:methanol (1:1), 1 ml diethyl ether:ethanol (1:1) and 1 ml diethyl ether. The protein (100-150  $\mu\text{g}$ ) pellet was dissolved in SDS-solution (6) and applied to slab gels for SDS-PAGE. Complete dissolution of the proteins in the SDS-cocktail, as verified by the absence of labeled material on the top of the separating gel, was critically dependent on repeated heating of the samples to  $96^{\circ}$ , sonication in a sonifying bath and Vortex mixing during at least a 3 h period. The samples were then immediately applied to the gel. After electrophoresis (6) stained and dried gels were autoradiographed on Kodak X-Omat R films for about 5 h, using an intensifier screen (DuPont). The  $^{32}\text{P}$ -radioactivity in the HSL band cut out in a gel slice was quantitated by liquid scintillation counting after correction for background in a gel slice of corresponding size from the 100-110 k are where no [ $^{32}\text{P}$ ]phosphopeptide band was visible (Fig. 1). No significant variation in quenching was found between samples. The relative  $^{32}\text{P}$  content could also be estimated by scanning densitometry of the autoradiograph, as the peak height of the HSL band above the base-line in the 100-110 k area of the sample track. There was a linear correlation between the results obtained with these two methods. Values given are always the mean of two samples. The coefficient of variation for duplicates was approx. 8% for the basal phosphorylation and 6% after maximal noradrenaline stimulation.

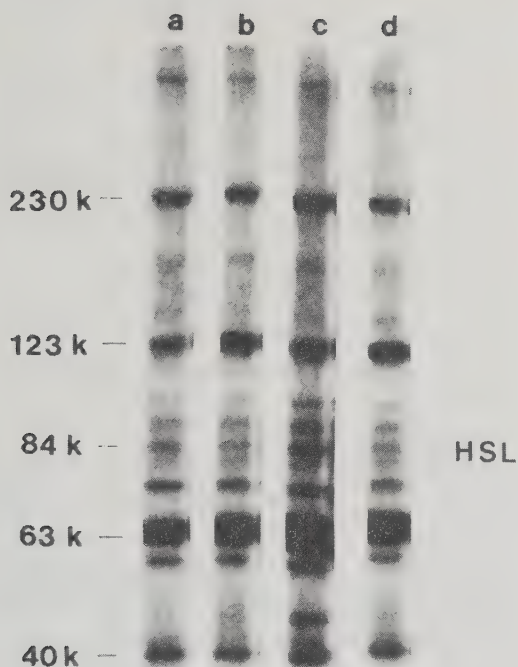
### Determination of lipolysis rate

Both FFA and glycerol release reflects lipolysis rate since reesterification of released fatty acids was insignificant under the experimental conditions used (*cf.* 3). The term 'lipolysis rate' will therefore be used synonymously for these parameters. FFA release from adipocytes was measured continuously by pH-stat titration as in (3,7). For simultaneous registration of lipolysis in controls and after addition of hormones and other agents two identical pH-stat titration systems were connected to a dual channel recorder. Rates of lipolysis in both incubations were recorded, after direct derivation, on this recorder (7). The incubation volume used (10-15 ml) allowed multiple sampling for [ $^{32}$ P]HSL analysis during lipolysis monitoring (values given have been corrected for volume changes). Glycerol release into the medium was analyzed by an enzymatic fluorometric method (10) modified for higher sensitivity as in (11).

### Other analytical procedures and materials

Intracellular ATP was quantitated in perchloric acid extracts of floated cells by an enzymatic fluorometric assay (12). [ $^{32}$ P]ATP radioactivity in the extract was assayed after thin layer chromatography on poly(ethyleneimine)cellulose (13).

[ $^{32}$ P]orthophosphate and [ $3\text{-}^3\text{H}$ ]glucose was from the Radiochemical Centre, Amersham, collagenase (CLS, batch 49K104) from Millipore Corporation, Freehold, N.J. Bovine serum albumin fraction V (dialysed for 4 x 6 h against distilled water and filtered through 0.8  $\mu\text{m}$  Millipore filters) was from Sigma Chemical Co., as was 1-noradrenaline bitartrate, N<sup>6</sup>-O<sup>2</sup>-dibutyryl cyclic adenosine 3'-5'-monophosphate (db-cAMP or dibutyryl-cAMP) and adenosine. Adenosine deaminase (E.C. 3.5.4.6) and enzymes for ATP analysis were from Boehringer Mannheim. Insulin (monocomponent porcine insulin, essentially glucagon-free, 1 pM=0.145  $\mu\text{U/ml}$ ) was a generous gift from Novo Laboratories, Copenhagen. Acrylamide and bisacrylamide was from Bio-Rad. All other reagents were also highest quality available commercially.



**Fig. 1.** Effects of hormones on adipocyte [ $^{32}\text{P}$ ]phosphopeptides separated by SDS-PAGE. Adipocytes (50  $\mu\text{l}$  PCV/ml) were incubated in modified Krebs-Ringer medium (see METHODS) with 3.5% bovine serum albumin and [ $^{32}\text{P}$ ]orthophosphate (50  $\mu\text{Ci/ml}$ , 50  $\mu\text{M}$  phosphate) for 40 min and hormones then added. The adipocyte proteins (approx. 150  $\mu\text{g/sample}$ ) were defatted, dissolved in SDS, fractionated by SDS-PAGE and autoradiographed (see METHODS). (a) no hormone addition; (b) insulin, 700 pM, for 5 min; (c) noradrenaline, 500 nM for 5 min; (d) noradrenaline, 500 nM for 5 min followed by insulin, 700 pM, for 5 min. Apparent  $M_r$  was estimated from the positions of rabbit muscle phosphorylase  $\alpha$  (94,000 = 94 k), human transferrin (76.7 k), bovine serum albumin (67 k), catalase from bovine liver (60 k) and ovalbumin (43 k). HSL = [ $^{32}\text{P}$ ]hormone-sensitive lipase.

## RESULTS

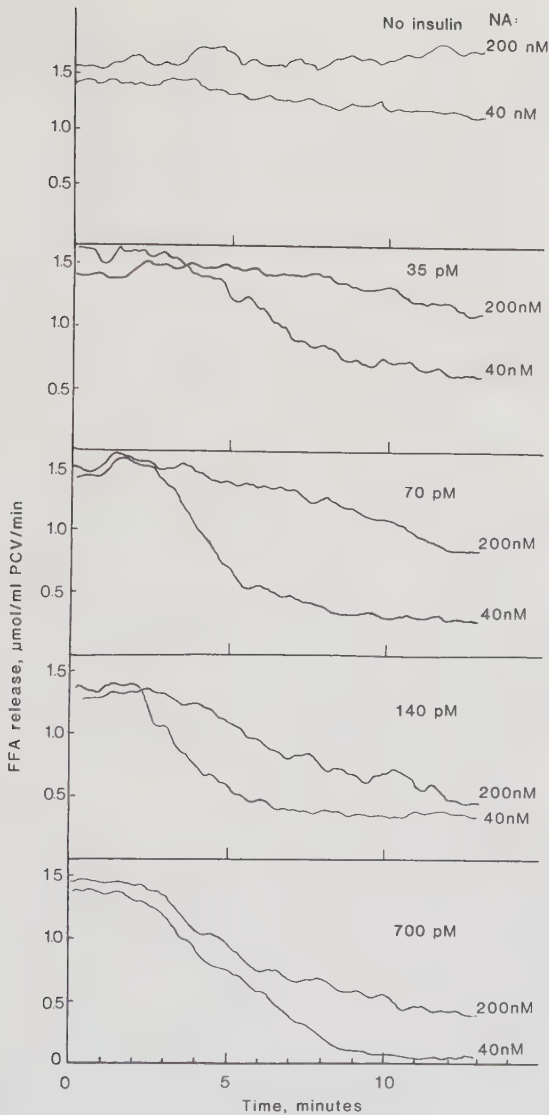
Basal  $^{32}\text{P}$ -phosphorylation of hormone-sensitive lipase in non-stimulated adipocytes

Incubation of rat adipocytes with [ $^{32}\text{P}$ ]orthophosphate caused a slow increase of the intracellular [ $^{32}\text{P}$ ]ATP specific activity to a steady state level after 30-40 min of incubation, with no significant changes of this parameter ( $< 10\%$  increase) over at least the subsequent 30 min (data not illustrated, *cf.* 6). Addition of noradrenaline (500 nM), insulin (700 pM) or glucose (0.55 mM) did not significantly change the specific [ $^{32}\text{P}$ ]ATP-radioactivity, thus changes of [ $^{32}\text{P}$ ]phosphopeptide radioactivity after 40 min preincubation reflect net changes of extent of phosphorylation.

The increase of [ $^{32}\text{P}$ ]ATP specific radioactivity was accompanied by an increase of [ $^{32}\text{P}$ ]HSL to a steady state level after 40 min incubation (*cf.* 6 and Fig. 1a,3A) with no measurable change of the lipolysis rate. This  $^{32}\text{P}$ -phosphorylation of the enzyme will be referred to as basal  $^{32}\text{P}$ -phosphorylation. It is likely to be due mainly to phosphorylation at another site(s) than that (those) involved in the hormonal regulation of the enzyme activity (see below) and also to [ $^{32}\text{P}$ ]phosphate exchange without net increase in the extent of phosphorylation. In the following, effects of hormones on the  $^{32}\text{P}$ -phosphorylation of the enzyme (Fig. 1c,d) will be expressed relative to this basal [ $^{32}\text{P}$ ]HSL value when so indicated.

Effects of insulin on the extent of hormone-sensitive lipase phosphorylation and lipolysis rate in adipocytes stimulated with noradrenaline

Adipocytes were stimulated with a maximal (200 nM) and sub-maximal (40 nM) concentration of noradrenaline and insulin added after 5 min preincubation (Fig. 2). The noradrenaline-stimulated lipolysis was inhibited by insulin in a concentration-dependent manner with a measurable insulin effect after a time lag which decreased from approx. 4 min to 2 min with increasing insulin concentration, and with full effects after



**Fig. 2.** Effects of different concentrations of insulin (0-700 pM) on the time-course of FFA release from adipocytes stimulated with 40 or 200 nM noradrenaline (NA). Adipocytes (10  $\mu$ l PCV/ml) were incubated in modified Krebs-Ringer medium with 10 mg/ml albumin in pH-stat cells for continuous measurement of FFA release. All experiments were performed under the same conditions with one cell batch. Insulin was added at zero time = 10 min after the addition of noradrenaline. (The concentration of insulin is indicated above each diagram and that of noradrenaline after the corresponding curve).



3-12 min. As expected, the insulin effects were more pronounced after submaximal than after maximal noradrenaline stimulation and full effects were also obtained more quickly. Sensitivity to insulin was less in these relatively dilute cell suspensions (10  $\mu$ l PCV/ml) than in more dense suspensions (50  $\mu$ l PCV/ml) (Fig. 4) in which endogenously produced adenosine could be assumed to accumulate to a greater extent (see below).

Insulin (700 pM) added with the [ $^{32}$ P]orthophosphate during the 40 min preincubation period did not significantly change the basal  $^{32}$ P-phosphorylation of HSL (8% decrease in four separate experiments, difference not significant) or the time required to obtain a steady-state [ $^{32}$ P]HSL level. Adenosine also had little effect on this basal  $^{32}$ P-phosphorylation of HSL (less than 10% decrease). Insulin (700 pM) added when a constant [ $^{32}$ P]HSL level had been obtained (after 40 min preincubation) also had no significant effects on the extent of basal  $^{32}$ P-phosphorylation of HSL or lipolysis (Fig. 1b,3A). These results indicate that the basal HSL phosphorylation mainly occurred at another phosphorylatable site(s) than that (those) involved in the hormonal regulation of the HSL activity, and thus of the lipolysis rate.

Insulin (700 pM) completely prevented the rapid net increase of [ $^{32}$ P]HSL and lipolysis after stimulation with 100 nM noradrenaline (Fig. 3B). If added after maximal noradrenaline effects had occurred insulin again decreased both parameters to basal levels (Fig. 1c,d; Fig. 3C). As described previously (6) the insulin effect on the noradrenaline-stimulated [ $^{32}$ P]-HSL level preceded its effect on the lipolysis rate, indicating a causal relationship between the two parameters. This was also clearly demonstrated when the inhibition of maximally (200 nM) noradrenaline-stimulated lipolysis by insulin and its corresponding effect on the extent of [ $^{32}$ P]HSL phosphorylation were related to insulin concentration in parallel experiments under identical conditions (Fig. 4). The antilipolytic effect was measured as the net decrease of noradrenaline-stimulated glycerol release over a 15 min incubation period from the addition of insulin, thus the inhibition is less in

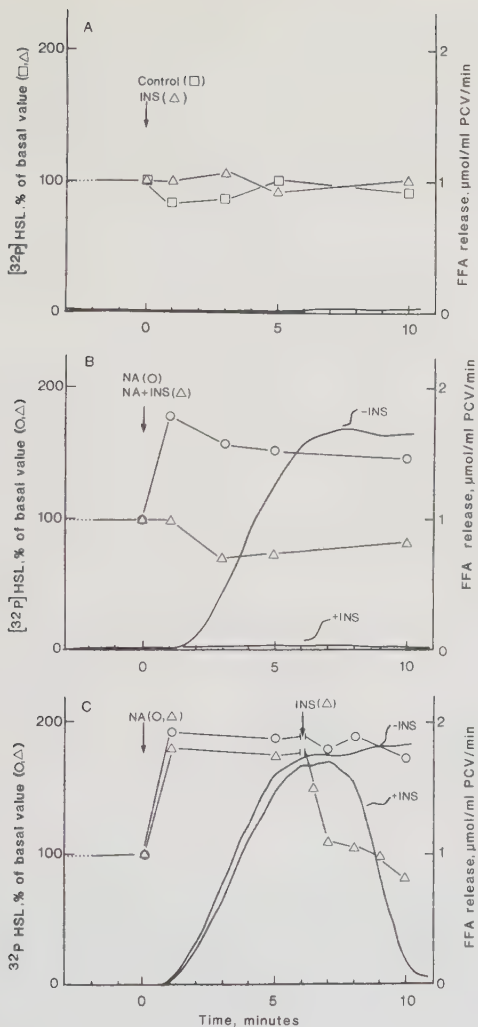


Fig. 3. Effect of insulin on the time-course of  $^{32}\text{P}$ -phosphorylation of HSL and lipolysis rate in control and noradrenaline-stimulated adipocytes incubated as in Fig. 1. The rate of FFA release was measured by pH-stat titration and samples were analyzed for  $^{32}\text{P}$ HSL. (A) Effect of 700 pM insulin compared to control without insulin; (B) Effect of 700 pM insulin added together with 0.1  $\mu\text{M}$  noradrenaline compared to noradrenaline control; (C) Effect of 700 pM insulin added after 0.1  $\mu\text{M}$  noradrenaline compared to noradrenaline control. Controls and experiments with hormones were performed with the same cell batch in parallel pH-stat incubations (see METHODS). The  $^{32}\text{P}$ HSL values are related to the basal phosphorylation obtained after 40 min incubation with  $^{32}\text{P}$ orthophosphate and are the means of two determinations from a representative experiment. Arrows indicate additions of hormones, NA = noradrenaline, INS = insulin.

absolute terms than the insulin effects on lipolysis rate obtained after 8-10 min (*cf.* Fig. 2). On the other hand, the cell suspensions were more dense (50  $\mu$ l PCV/ml) with presumably more accumulated adenosine acting synergistically with insulin (see below) and increasing the apparent sensitivity to the hormone (*cf.* Fig. 2) with half-maximal inhibition at 20-30 pM.

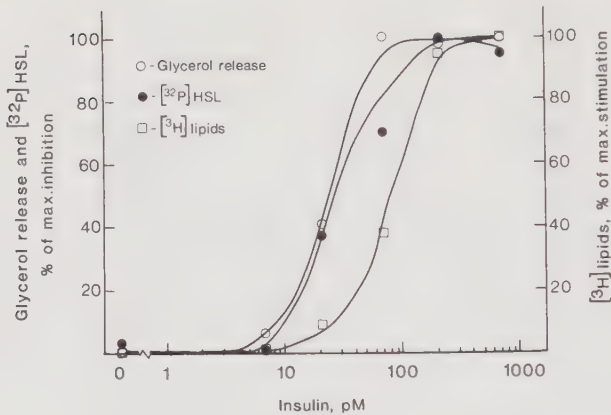
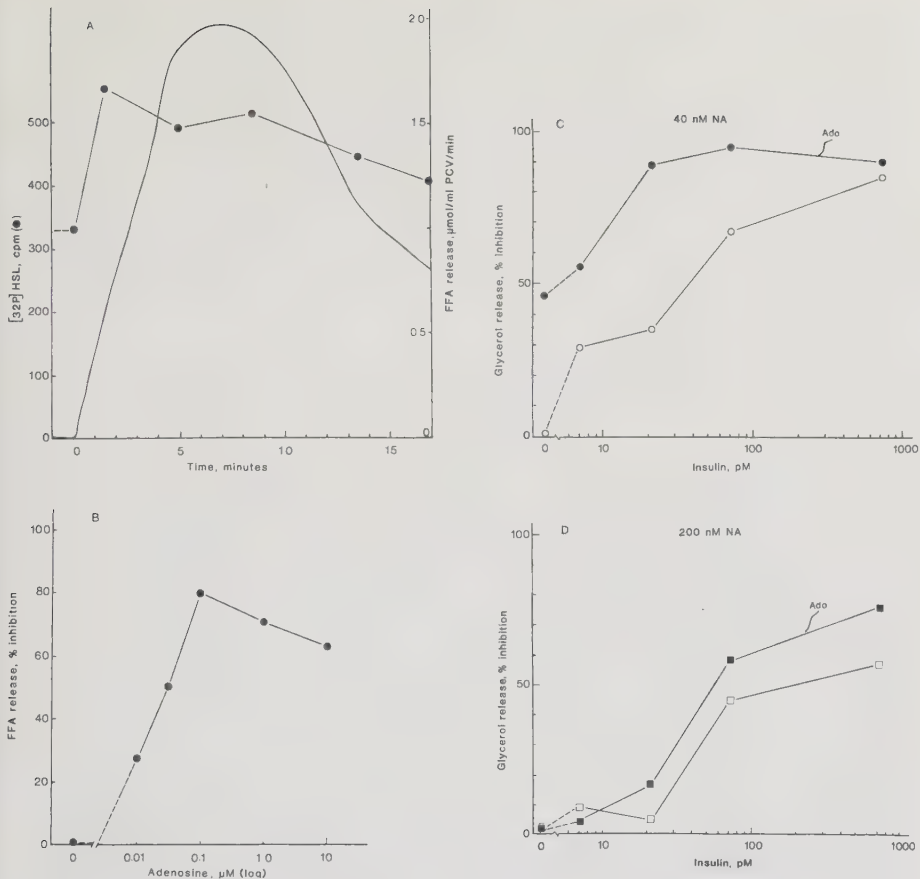


Fig. 4. Effects of varying insulin concentrations on the inhibition of lipolysis, extent of  $^{32}\text{P}$ -phosphorylation of HSL, and on glucose conversion to cell lipids in adipocytes stimulated with noradrenaline. Three samples of cells were incubated identically (50  $\mu$ l PCV/ml in modified Krebs-Ringer medium with 3.5% albumin, 0.55 mM glucose and 50  $\mu\text{M}$   $\text{P}_i$ ) for each insulin concentration except for additions of carrier-free radio isotopes when appropriate. After a 40 min incubation 200 nM noradrenaline was added followed 5 min later by varying concentrations of insulin. Glycerol release and conversion of [ $^3\text{H}$ ]glucose into cellular lipids was measured over 15 min following insulin addition, and [ $^{32}\text{P}$ ]HSL was determined at the end of that incubation period. The values are based on duplicate samples obtained in each one of two separate experiments. With 700 pM insulin, the glucose incorporation increased 19-fold, the glycerol release was inhibited by 62%, and the  $^{32}\text{P}$ -phosphorylation of HSL was inhibited by 40%, compared to the values obtained in the absence of insulin.



**Fig. 5.** Adenosine modification of the lipolytic response and <sup>32</sup>P-phosphorylation of HSL. A) Effects of adenosine deaminase on the extent of <sup>32</sup>P-phosphorylation of HSL and on lipolysis rate. Adipocytes (50 μl PCV/ml) were incubated as in Fig. 1 and FFA release was monitored by pH-stat titration. Adenosine deaminase, 1 μg/ml, was added at zero time = 40 min after addition of [<sup>32</sup>P]orthophosphate. B) Inhibition of noradrenaline-stimulated lipolysis by varying concentrations of adenosine (0-10 μM). Adipocytes (10 μl PCV/ml) were incubated as in Fig. 2, after resuspension in fresh medium, and lipolysis was measured by pH-stat titration. After 10 min incubation noradrenaline, 30 nM, was added, followed by the indicated concentrations of adenosine 7 min later. The plotted values express the percentage inhibition of FFA release rate due to adenosine after 5 min incubation with this agent. C,D) Effects of adenosine on the antilipolytic action of insulin in cells stimulated with (C) 40 nM and (D) 200 nM noradrenaline. Adipocytes (10 μl PCV/ml) in modified Krebs-Ringer medium with 24 mM HEPES and 35 mg/ml albumin, were incubated in the presence of noradrenaline for 5 min, before insulin at the indicated concentrations with or without 1 μM adenosine was added. The plotted values (mean of triplicates) express the percentage inhibition of glycerol release over 15 min following addition of insulin or this hormone in combination with adenosine. The rate of glycerol release with noradrenaline alone was 0.47 (40 nM NA) and 0.52 (200 nM NA) μmol/ml PCV/min.

In an attempt to relate the antilipolytic effect to another important insulin action the conversion of [ $^3\text{H}$ ]glucose to adipocyte lipids was measured in parallel experiments with the same cell batch under identical conditions (Fig. 4). This parameter mainly reflects cellular glucose uptake (9) and has been reported to be similarly (14,15) or less (2,16) sensitive to insulin concentration. The half-maximal stimulation obtained at 80-90 pM of insulin in the present experiments clearly supports the latter findings.

#### Modification by adenosine of the antilipolytic effect of insulin

Under the incubation conditions used in the experiments above endogenously produced adenosine, a well-known inhibitor of adenylate cyclase in fat cells (17), could be expected to accumulate and modify the results (18), especially when more concentrated cell suspensions were incubated for longer times as in the  $^{32}\text{P}$ -labelling experiments. To evaluate how this would influence the interpretation of the insulin effects several experiments were performed. Adenosine deaminase degrades adenosine, also that bound to the adipocyte plasma membranes (17), to inosine and thus diminishes or prevents the adenosine effect. Addition of adenosine deaminase to adipocytes prelabelled with [ $^{32}\text{P}$ ]orthophosphate caused a marked but transient increase of [ $^{32}\text{P}$ ]HSL and of the lipolysis rate (Fig. 5A) which indeed indicated the presence of an inhibitory concentration of adenosine in the system. Lipolysis rate in a more dilute suspension of adipocytes in fresh incubation medium (to remove accumulated adenosine) stimulated with submaximal noradrenaline was inhibited by adenosine added 7 min after the hormone, in a dose-dependent manner, with half-maximal effect at approx. 30 nM adenosine (Fig. 5B). In experiments reported elsewhere adenosine deaminase has been shown to increase markedly the cyclic AMP level obtained after maximal noradrenaline stimulation (19). However, this effect of the adenosine deaminase caused only slight additional increase of  $^{32}\text{P}$ -phosphorylation of HSL and did not further increase the rate of lipolysis (data not shown).

Addition of 1  $\mu\text{M}$  adenosine markedly potentiated the antilipolytic effect of insulin on submaximally (40 nM) noradrenaline-stimulated adipocytes (Fig. 5C), but had less effect on insulin inhibition of the lipolysis stimulated maximally (200 nM) (Fig. 5D) with noradrenaline. Thus, at least with submaximally stimulated lipolysis endogenously produced adenosine must be considered as an important modifier of the quantitative antilipolytic effects of insulin.

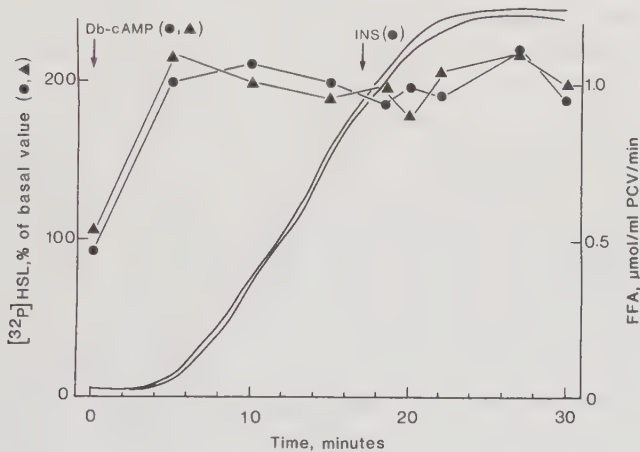


Fig. 6. Effects of dibutyryl cyclic AMP in the presence and absence of insulin on the extent of  $^{32}\text{P}$ -phosphorylation of HSL and on lipolysis rate in adipocytes (50  $\mu\text{l}$  PCV/ml) incubated as in Fig. 3. Arrows indicate the additions of 1 mM dibutyryl cyclic AMP (Db-cAMP) and 3.5 nM insulin (INS).  $[^{32}\text{P}]\text{HSL}$  expressed as percent of basal  $^{32}\text{P}$ -phosphorylation after 40 min incubation.

#### Effects of insulin on dibutyryl cyclic AMP-stimulated lipolysis

Dibutyryl cyclic AMP stimulated lipolysis rate, preceded by an increase of the extent of  $^{32}\text{P}$ -phosphorylation of HSL. Maximal dibutyryl cyclic AMP stimulation (1 mM) increased  $[^{32}\text{P}]\text{HSL}$  and the lipolysis rate (Fig. 6) to the same level as maximal noradrenaline stimulation (Fig. 3). Insulin, even at a very high concentration (3.5 nM) had no measurable effect on either the  $[^{32}\text{P}]\text{HSL}$  level or the lipolysis rate induced by maximal dibutyryl cyclic AMP (Fig. 6). However, slight and transient decreases of both these parameters were observed with submaxi-



mal dibutyryl cyclic AMP stimulation (data not shown). The time-course of dibutyryl cyclic AMP stimulation was different from that of maximal noradrenaline stimulation. There was at least a 2-3 min delay before [ $^{32}$ P]HSL started to increase (data not shown) and the increase of the lipolysis rate was also delayed in relation to the effect on [ $^{32}$ P]HSL (*cf.* Fig. 3B,C *vs.* Fig. 6). This may indicate additional effects of dibutyryl cyclic AMP besides increasing cellular cyclic AMP (20).

## DISCUSSION

Intact rat fat cells provide a convenient model to investigate regulation of adipose tissue lipolysis. The fat cells can easily be prepared and remain highly sensitive to various lipolytic agents and insulin over several hours. The pH-stat titration technique is an extraordinarily simple and convenient system for continuous monitoring of the lipolysis rate in the system, enabling detailed information on the onset and duration of action of each hormone or other agent. The [ $^{32}$ P]-HSL data provide direct information on the hormonal effects on the phosphorylation state of this rate-controlling enzyme.

The overall most significant finding of the present work was the close relationship between the phosphorylation state of the hormone-sensitive lipase ([ $^{32}$ P]HSL level related to the basal phosphorylation level) and the lipolysis rate. These two parameters correlated well under all conditions tested, both with lipolytic agents (noradrenaline, dibutyryl cyclic AMP, adenosine deaminase) and when lipolysis was inhibited with insulin or adenosine, and there was temporal as well as quantitative correlation. The dose-dependent, antilipolytic effect of insulin, readily demonstrated in the pH-stat titration system (Fig. 2), may thus be explained by net decrease of the extent of HSL phosphorylation. Moreover insulin added shortly before or together with noradrenaline completely prevented increased HSL phosphorylation and HSL activity, *i.e.* lipolysis rate (Fig. 3). From the present results it cannot be distin-

guished if the effects of insulin were mediated by changes in the rate of phosphorylation *i.e.* protein kinase activity or dephosphorylation, *i.e.* protein phosphatase activity. However, as discussed elsewhere (19) at least part of the insulin effect may have been due to prevention of the transient cellular cyclic AMP increase caused by noradrenaline stimulation of the fat cells.

The higher insulin concentration (Fig. 4) required to obtain half-maximal inhibition compared to that found previously (6), approx. 20 pM compared to approx. 6 pM was mainly due to differences in incubation conditions especially the higher noradrenaline concentration in the latter experiment.

Glucose conversion to adipocyte lipids, a process which is probably rate-limited by glucose uptake over the fat cell plasma membrane (9) required three- to fourfold higher insulin concentrations for half-maximal effect (Fig. 4). This was in contrast to the findings in a recent work (14) in which the stimulated lipogenic response to insulin was not significantly different from the sensitivity of the antilipolytic response to insulin. However, the comparison in that work (14) was performed under non-identical incubation conditions; fat cell concentration was severalfold higher when lipolysis was measured, and the cells in the glucose conversion experiment had not been stimulated with catecholamine. Moreover, half-maximal stimulation of lipolysis with adrenaline did not occur until at 2000 nM, giving a maximal glycerol release rate which could be estimated to be approx. 0.3  $\mu\text{mol/min/ml}$  PCV (assuming 1  $\mu\text{l}$  PCV =  $1.6 \cdot 10^4$  cells). Thus, the cells had markedly lower sensitivity to catecholamine stimulation and maximal lipolytic response than the cells used in the present work.

In initial experiments we generally found that the antilipolytic effect of insulin was more pronounced in concentrated fat cell suspensions ( $> 5 \cdot 10^5$  cells/ml) than in more dilute ones ( $10^5$  cells/ml), especially when the cells had been subjected to long preincubations. An explanation to this was

offered by the findings of the effects of adenosine deaminase and adenosine in the incubation system (Fig. 5). As could be expected from work by others endogenous adenosine produced by the cells probably accumulated on the cell plasma membrane and in the incubation medium (17,18). Adenylate cyclase inhibition by this adenosine (17) leading to inhibition of HSL phosphorylation and lipolysis rate, will quantitatively modify responses to insulin depending on the experimental conditions.

The experiments with dibutyryl cyclic AMP indicated that effects on HSL phosphorylation and lipolysis equivalent to those obtained with maximal noradrenaline could be caused by a direct increase of cellular cyclic AMP (*cf.* 20). Thus, there is no need to postulate an additional, separate noradrenaline-stimulated "substrate activation" process (21,22) to explain the lipolysis rate obtained by maximal stimulation with this hormone. In spite of the up to 50-fold increase of cellular cyclic AMP reported to result from millimolar concentrations of dibutyryl cyclic AMP (20), insulin even in high concentration did not inhibit HSL phosphorylation and lipolysis rate under these conditions. One possible explanation to these findings would be that the effects of insulin on HSL phosphorylation and lipolysis rate were mainly due to a net decrease of cellular cyclic AMP, *i.e.* direct reversal of noradrenaline stimulation. However, in view of the several actions of dibutyryl cyclic AMP which are not well understood (20) the present data certainly do not exclude additional cyclic AMP-independent insulin effects (*cf.* 19).

## ACKNOWLEDGEMENT

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THE ANTI-LIPOLYTIC EFFECT OF INSULIN:  
TIME-COURSE RELATIONSHIPS BETWEEN  
CYCLIC ADENOSINE 3':5'-MONOPHOSPHATE LEVELS  
PHOSPHORYLATION STATE OF HORMONE-SENSITIVE LIPASE  
AND LIPOLYSIS IN RAT ADIPOCYTES

By

Nils Östen Nilsson, John Fain\* and Per Belfrage  
Department of Physiological Chemistry 4, University of Lund,  
Lund, Sweden and \*Division of Biology and Medicine  
Brown University, Providence, R.I., U.S.A.

## SUMMARY

The anti-lipolytic effect of insulin was studied in intact rat adipocytes by relating cyclic AMP concentration to the phosphorylation state of hormone-sensitive lipase (HSL) and to the lipolysis rate. Maximal noradrenaline stimulation of the HSL phosphorylation state and lipolysis rate in the concentrated fat cell suspensions used was associated with a twofold transient increase of cyclic AMP, which returned to the basal level within five minutes. Increasing the cyclic AMP levels with theophylline caused no further change of the parameters studied. The data were consistent with  $\beta$ -adrenoceptor stimulation of lipolysis through cyclic AMP-mediated, increased HSL phosphorylation. These results and data from experiments with the  $\beta$ -blocking agent propranolol indicated that hormonal control of cyclic AMP was compartmentalized.

Insulin prevented most of the noradrenaline-induced increase of cyclic AMP and blocked HSL phosphorylation, indicating this to be an important mechanism for the antilipolytic action of the hormone. However, comparison with the effects from  $N^6$ -(phenylisopropyl)adenosine, a potent inhibitor of adenylate cyclase and cyclic AMP production, under several experimental conditions, suggested that insulin inhibited noradrenaline-stimulated lipolysis also by an additional cyclic AMP-independent mechanism(s).

Abbreviations used are: HSL, hormone-sensitive lipase; cyclic AMP, cyclic adenosine 3':5'-monophosphate; FFA, free fatty acids; PIA,  $N^6$ -(phenylisopropyl)adenosine; PCV, packed cell volume; NA, noradrenaline; INS, insulin; Theo, theophyllin; Prop, propranolol; Ado.dea, adenosine deaminase; SDS-PAGE, polyacrylamidegelelectrophoresis in presence of sodium dodecylsulphate.

## INTRODUCTION

Previous work has established that noradrenaline stimulates triacylglycerol lipolysis in intact rat adipocytes by increasing the extent of phosphorylation of hormone-sensitive lipase (HSL) (1), the rate-controlling enzyme in this catabolic process. Insulin exerted its antilipolytic effect by preventing or decreasing the noradrenaline-stimulated increase of the HSL phosphorylation state (1,2).

Catecholamine stimulation of adipose tissue lipolysis is believed to be mediated through cyclic AMP increase and activation of cyclic AMP-dependent protein kinase (3,4). This postulated mechanism is supported by the finding that cyclic AMP-dependent protein kinase catalyzed the phosphorylation of isolated HSL, resulting in its activation (5). Moreover, incubation of intact rat adipocytes with dibutyryl cyclic AMP, which would lead to a marked increase of intracellular cyclic AMP (6), stimulated HSL phosphorylation and lipolysis rate to the same extent as that obtained by maximal noradrenaline stimulation (2).

In contrast to this the mechanism by which insulin prevents or causes net decrease of noradrenaline-stimulated HSL phosphorylation, and thereby inhibits lipolysis (1,2), is far from clear. The role of cyclic AMP in this process is highly controversial (4); both cyclic AMP-dependent and -independent mechanisms for the insulin action have been proposed (4,7,8).

The newly developed technique for determination of the phosphorylation state of HSL in intact rat adipocytes (1,2) prompted us to relate insulin effects on this parameter to cyclic AMP levels and the lipolysis rate in an attempt to further clarify the mechanism for the antilipolytic action of insulin. The results indicate that decreased cellular cyclic AMP is an important part of this mechanism, but also suggest that an additional, cyclic AMP-independent effect(s) occurs.

## MATERIALS AND METHODS

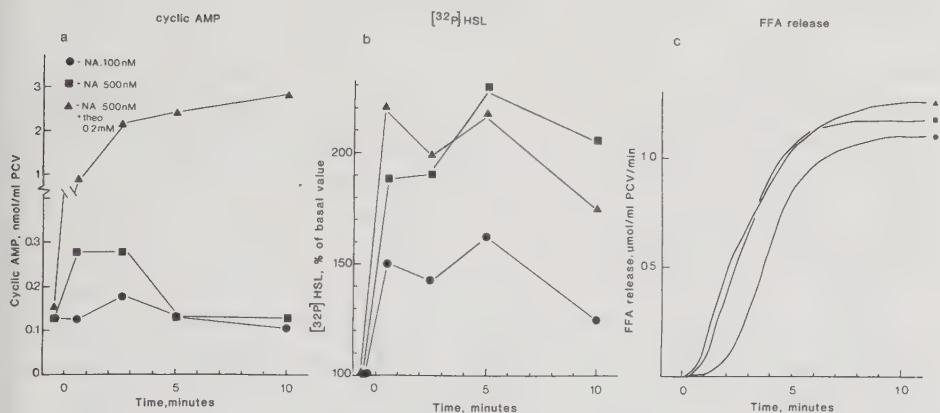
Adipocytes were prepared by collagenase digestion (collagenase CLS, batch 49K104, Millipore Corporation, Freehold, NJ, U.S.A.) of adipose tissue from 120-150 g male Sprague-Dawley rats as in (9). Cell viability was evaluated as in (9) and cell concentration was determined and expressed as packed cell volume (PCV), where 1  $\mu$ l PCV corresponds to an average of  $1.6 \cdot 10^4$  cells. The adipocytes were incubated in modified Krebs-Ringer (131 mM NaCl; 4.95 mM KCl; 2.54 mM  $\text{CaCl}_2$ ; 1.19 mM  $\text{MgSO}_4$  (9)) with 50  $\mu$ M sodium phosphate (with or without 50  $\mu$ Ci/ml carrier-free [ $^{32}$ P]orthophosphate) and 35 mg/ml bovine serum albumin in two 10 ml pH-stat vessels, from which samples (0.5 ml) were withdrawn for analysis of cyclic AMP (from an incubation without [ $^{32}$ P]orthophosphate) and extent of phosphorylation of HSL (from an incubation with [ $^{32}$ P]orthophosphate). Lipolysis was monitored continuously in the same incubations by pH-stat titration of FFA release (9). Under the conditions of the experiments insignificant reesterification took place; lipolysis rate was therefore directly measured by the FFA release rate.

Cyclic AMP samples (duplicates) were promptly treated with 50  $\mu$ l 2 M HCl, heated to 100°C for 1 min and neutralized. Cyclic AMP was then determined in triplicate on each sample by the protein kinase binding assay of Gilman (10). The values for cyclic AMP given in the Figures are the means; the variation coefficient varied from approx. 20% (basal levels) to 6% (maximally stimulated values).

[ $^{32}$ P]HSL (duplicates) was determined after separation of defatted total cell proteins by SDS-PAGE as in (1,2). The data plotted are the means; the variation coefficient was less than 10%.

Due to differences between cell batches data from individual representative experiments have been plotted rather than combined data from several different cell preparations.

Carrier-free [ $^{32}$ P]orthophosphate was from the Radiochemical Centre, Amersham, L-noradrenaline bitartrate, D,L-propranolol and theophylline from Sigma Chemical Co, St. Louis, U.S.A., insulin (monocomponent pork insulin, 1 pM = 0.145  $\mu$ U/ml) from Novo Laboratories, Copenhagen, and N<sup>6</sup>-(phenylisopropyl)-adenosine and adenosine deaminase (200 U/mg) (E.C. 3.5.4.6) from Boehringer, Mannheim, Germany.



**Fig. 1.** Effects of varying concentrations of noradrenaline and noradrenaline plus theophylline on the time-course of (a) the cyclic AMP level (cells + medium), (b) the extent of  $^{32}$ P-phosphorylation of hormone-sensitive lipase ([ $^{32}$ P]HSL), and (c) the lipolysis rate. Isolated rat adipocytes (50  $\mu$ l PCV/ml) were incubated in modified Krebs-Ringer medium (see METHODS) with 35 mg/ml bovine serum albumin and 50  $\mu$ M sodium orthophosphate combined with carrier-free [ $^{32}$ P]orthophosphate (50  $\mu$ Ci/ml) when appropriate. Zero time is after a 40 min preincubation. Incubations were performed in two identical pH-stat vessels, one for [ $^{32}$ P]HSL determinations and one without [ $^{32}$ P]orthophosphate for cyclic AMP analysis. FFA release rate in both incubation vessels was monitored by pH-stat titration and did not significantly differ between the two incubations (illustrated by continuous lines). All experiments were performed with the same cell batch.



## RESULTS

Time-course of noradrenaline-effects on cyclic AMP concentration, HSL phosphorylation state and lipolysis rate

Parallel incubations in two pH-stat titrator vessels made it possible to determine cyclic AMP concentration, extent of HSL  $^{32}\text{P}$ -phosphorylation and lipolysis rate under identical incubation conditions, at short intervals after addition of noradrenaline alone, or combined with theophylline to allow cyclic AMP to accumulate. The results obtained (Fig. 1a,b,c) are consistent with a causal interrelationship between the cyclic AMP increases and HSL phosphorylation/lipolysis. However, the relation between these parameters was obscured by several findings. Maximal HSL phosphorylation and lipolysis were obtained by two-fold increase of the cyclic AMP concentration and half-maximal or more by some 25% increase above the basal level. The tenfold higher cyclic nucleotide concentration obtained with theophylline caused no additional effects. The increase of cyclic AMP was transient unless theophylline was included and values returned to the basal level within 5 min, while the extent of phosphorylation of HSL and especially the lipolysis rate remained high. With submaximal (100 nM) noradrenaline HSL phosphorylation had almost reached its highest level after 0.5 min although no increase of cyclic AMP could be measured at this time point. Thus, it was evident that the increase of HSL phosphorylation and lipolysis rate were poorly related to the measured total cyclic AMP concentration. The possibility that they were more directly related to a sub-cellular pool of cyclic AMP will be discussed below.

The lack of direct relation between total cyclic AMP and HSL phosphorylation/lipolysis rate was also found when cyclic AMP production was blocked with the non-selective  $\beta$ -adrenergic agonist, propranolol (Fig. 2). When this agent was added 5 min after the addition of 300 nM noradrenaline, HSL phosphorylation was rapidly decreased, followed by inhibition of the lipolysis rate. Since at this time cyclic AMP levels should have returned to the basal level (Fig. 1a) the propranolol effect

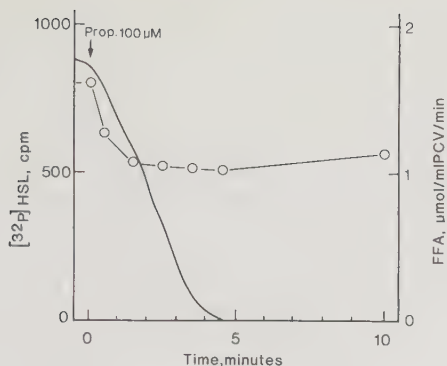
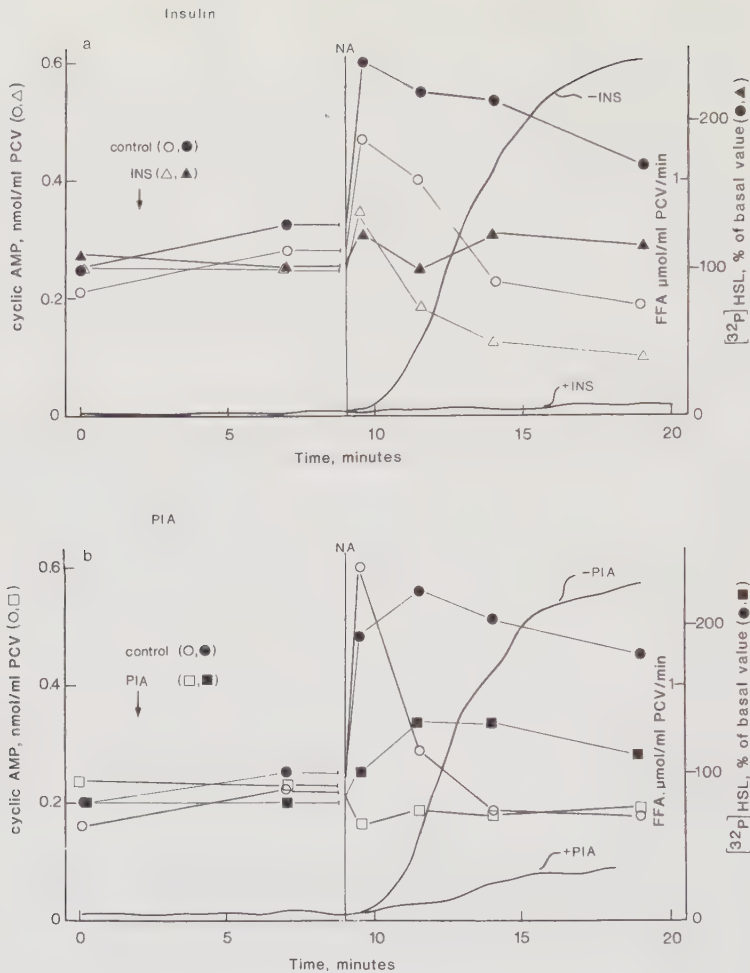


Fig. 2. Time-course of effects by propranolol, a nonselective  $\beta$ -blocking agent, on the extent of  $^{32}\text{P}$ -phosphorylation of HSL and on lipolysis rate in noradrenaline stimulated adipocytes. The incubation of cells (50  $\mu\text{l}$  PCV/ml) was performed as in Fig. 1. After 5 min incubation with 300 nM noradrenaline, 100  $\mu\text{M}$  propranolol (arrow) was added = zero time. Before noradrenaline addition the [ $^{32}\text{P}$ ]HSL value was 520 cpm (mean of duplicates) and the FFA release rate less than 0.03  $\mu\text{mol/ml}$  PCV/min.

must be explained in terms of decrease of a small but functionally important cyclic AMP pool, changes of which did not measurably affect the total cyclic AMP concentration.

#### Time-course of effects of insulin and phenylisopropyladenosine (PIA) on cyclic AMP concentration, HSL phosphorylation state and lipolysis rate in noradrenaline-stimulated adipocytes

In experiments of similar design as those described above the antilipolytic effect of insulin was compared with that of PIA, an adenosine analogue which would strongly inhibit adenylate cyclase and thus cyclic AMP production (11). In order to decrease endogenous adenosine which had accumulated in the incubations during the 40 min preincubation with [ $^{32}\text{P}$ ]orthophosphate the fat cells were transferred to fresh medium of the same composition a short time before additions of hormones. This caused a slight increase of the basal cyclic AMP level (from approx. 0.15 to 0.20-0.25 nmol/g PCV of fat cells) with no other measurable effects (*cf.* Fig. 1a with Fig. 3a,b). Insulin (700 pM), added before noradrenaline, at a maximally

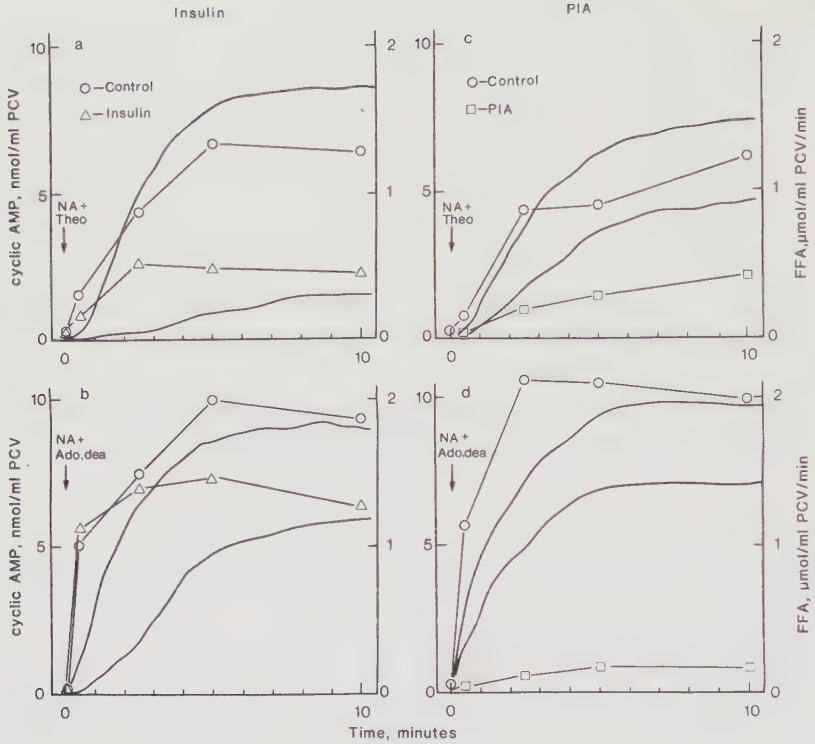


**Fig. 3.** Time-course of effects by insulin (a) and phenylisopropyladenosine (PIA) (b) on the noradrenaline stimulated cyclic AMP level, HSL  $^{32}\text{P}$ -phosphorylation and lipolysis rate. Adipocytes (50  $\mu\text{l}$  PCV/ml) were incubated and the parameters determined as in Fig. 1, except that the medium was changed 10 min before zero time in order to diminish endogenous adenosine produced during the  $[^{32}\text{P}]\text{orthophosphate}$  labelling period. Control incubations with noradrenaline alone were performed in parallel under identical conditions. Arrows indicate the addition of 700 pM insulin (INS) and 100 nM phenylisopropyladenosine, respectively, and the vertical line the addition of 500 nM noradrenaline. All experiments were performed with the same cell batch.

stimulating concentration (500 nM), almost completely prevented the noradrenaline-induced increase of the HSL phosphorylation state and lipolysis rate (Fig. 3a). The insulin addition prevented most of the transient cyclic AMP increase due to noradrenaline, indicating that reduction of cyclic AMP may indeed be an important mechanism for the insulin effect on HSL phosphorylation state and lipolysis rate. However, the reduction of cyclic AMP was not complete, a small initial increase was consistently found followed by decrease to a level below that found before the addition of noradrenaline. A different picture emerged in the experiments with PIA (Fig. 3b). In spite of the apparent complete prevention of noradrenaline-stimulated cyclic AMP increase by this agent it has considerably less effect on HSL phosphorylation state and lipolysis (Fig. 3b) than insulin.

Effects of insulin and PIA on cyclic AMP and lipolysis rate in adipocytes stimulated with noradrenaline in combination with theophylline or adenosine deaminase

In further attempts to describe the antilipolytic effect of insulin the effect of this hormone was compared with that of PIA in adipocytes incubated under such conditions that cyclic AMP would accumulate extensively. Insulin reduced cyclic AMP and inhibited lipolysis stimulated with maximal noradrenaline in combination with 0.2 mM theophylline (Fig. 4a). The antilipolytic effect of insulin was greater than that of PIA (Fig. 4c) although the effects on cyclic AMP were less pronounced. Similarly, insulin partially inhibited lipolysis caused by maximal noradrenaline in combination with adenosine deaminase although the reduction of cyclic AMP was small, or at early times, insignificant (Fig. 4). PIA, in contrast, under the same conditions caused less decrease of the lipolysis rate, although the reduction of cyclic AMP was much more pronounced (Fig. 4d).



**Fig. 4.** Time-course of effects by insulin (a,b) and phenylisopropyladenosine (PIA) (c,d) on the cyclic AMP level and lipolysis rate in adipocytes stimulated with noradrenaline + theophylline (a,c) and noradrenaline + adenosine deaminase (b,d). Adipocytes ( $50 \mu\text{l PCV/ml}$ ) were incubated as in Fig. 3. Zero time is after a 7 min preincubation with  $700 \text{ pM}$  insulin (a,b) or  $0.1 \mu\text{M}$  phenylisopropyladenosine (PIA) (c,d). Arrows indicate the addition of  $500 \text{ nM}$  noradrenaline (NA) plus  $0.2 \text{ mM}$  theophylline (Theo) (a,c), or  $500 \text{ nM}$  noradrenaline plus  $0.5 \mu\text{g/ml}$  of adenosine deaminase (Ado,dea) (b,d). All experiments were performed with the same cell batch.

## DISCUSSION

The results of this work provide strong evidence for the concept that the catecholamine activation of the  $\beta$ -adrenoceptors stimulates the lipolysis rate in intact adipocytes through increase of cellular cyclic AMP with subsequently increased HSL phosphorylation. Additional evidence for this activation mechanism has been obtained previously: isolated HSL was phosphorylated and activated by cyclic AMP-dependent protein kinase from rat adipose tissue (5) and incubation of intact rat adipocytes with dibutyryl cyclic AMP caused the same effects (2).

The data also indicate that the hormonal control of the cyclic nucleotide level was compartmentalized (*cf.* 12,13,14): The HSL phosphorylation state and the lipolysis rate remained high even after cyclic AMP apparently had returned to the basal level (Fig. 1). Moreover, propranolol (Fig. 2) or adenosine (2) inhibition of cyclic AMP production, at a time point when cyclic AMP would have returned to the basal level (*cf.* Fig. 1a), promptly decreased the HSL phosphorylation state and lipolysis. The only reasonable explanation to these findings is that the  $\beta$ -adrenoceptor activation increased a small, functionally important pool of cyclic AMP at the same time as an excess of non-functional (*i.e.* with no effect in the regulation of lipolysis) cyclic AMP started to accumulate, presumably at another subcellular compartment. The concentration of the functional pool of cyclic AMP, which may have constituted only a few percent of the total cyclic AMP, must then have remained at a high local concentration, maintaining a high activation state of cyclic AMP-dependent protein kinase and HSL. Agents which blocked cyclic AMP production (*e.g.* propranolol, adenosine) could thus have caused a lowering of this functional cyclic AMP pool too small to be detected as a change of total cyclic AMP. The decrease of the cyclic AMP, postulated to be nonfunctional, may have been caused by increased low  $K_m$  phosphodiesterase activity, *i.e.* increased degradation of the cyclic nucleotide. Catecholamine stimulation is known to rapidly increase the activity of this enzyme (15,16).



The time-course and magnitude of cyclic AMP accumulation after catecholamine stimulation is strongly dependent on the accumulation of endogenously produced adenosine in the incubation medium (17,18). The extent of adenosine release is mainly governed by the fat cell concentration and the preincubation time (17). In the present experiments, with a high cell concentration and long preincubation time, marked adenosine inhibition of adenylate cyclase and cyclic AMP can be expected to have taken place, as indicated by the close similarity between our results on cyclic AMP (Fig. 1) and those with concentrated cell suspensions in (18).

Insulin addition to intact rat adipocytes was previously found to prevent subsequent noradrenaline increase of the HSL phosphorylation state and of the lipolysis rate (2). The results of the present work (Fig. 3a) indicate that inhibition of noradrenaline-induced cyclic AMP increase may have been an important reason for this insulin effect. The mechanism for the reduction of cyclic AMP is likely to be the well-known stimulation by insulin of the low  $K_m$  phosphodiesterase activity (19,20). The lowered cyclic AMP would decrease cyclic AMP-dependent protein kinase activity, *i.e.* HSL phosphorylation. The lowered activity of this enzyme could also lead to increased HSL dephosphorylation through increased protein phosphatase activity mediated via a specific protein phosphatase inhibitor (21).

However, there are several indications of additional insulin effects. The small cyclic AMP peak consistently found immediately after noradrenaline addition (Fig. 3a) would have been large enough to be accompanied by a substantial increase of HSL phosphorylation and lipolysis, if elicited by submaximal noradrenaline stimulation alone (*cf.* Fig. 1a). In fact, apparently complete prevention of noradrenaline-induced cyclic AMP increase with PIA (Fig. 3b) caused less efficient inhibition of HSL phosphorylation and lipolysis than that obtained by insulin. Moreover, insulin had less effect than PIA on cyclic AMP levels but inhibited lipolysis more efficiently,

especially at early times, when fat cells were incubated with maximal noradrenaline plus theophylline or adenosine deaminase (Fig. 4a-d).

One possible explanation to these findings would be that insulin, besides reducing cyclic AMP, also affected the HSL phosphorylation state through a cyclic AMP-independent mechanism(s). It is conceivable that insulin, *e.g.* through postulated chemical mediators (22,23), could inhibit HSL phosphorylation via interference with cyclic AMP-dependent protein kinase (22), or increase HSL dephosphorylation via increased protein phosphatase activity (23). However, considering the evidence for compartmentalized hormonal control of cyclic AMP (see above) it cannot be excluded that all the insulin effects could have been mediated through reduction of a small functional cyclic AMP pool.

In view of the finding that insulin had little effect on the cyclic AMP level and the lipolysis rate in dilute adipocyte suspensions (18) the question of the physiological relevance of the present results may well be brought up. However, the model system used in this work has several features in common with intact adipose tissue. The basal and noradrenaline-stimulated levels were similar (24) and insulin has consistently been found to decrease cyclic AMP in intact adipose tissue (25-27). Thus, the marked adenosine inhibition of adenylate cyclase found in concentrated cell suspensions, like those used in the present work, may be a desirable property of the model system when studying the mechanism of insulin action in intact adipocytes.

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